Kinase Pathways in the Stabilization of a Postsynaptic Apparatus

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Dissertation

zur

Erlangung der naturwissenschaftlichen Doktorwürde

(Dr. Sc.nat)

Vorgelegt der

Mathematisch-naturwissenschaftlichen Fakultät

der

Universität Zürich

von

Gayathri Sadasivam

aus

Indien

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Zürich 2006

To my Father

(1942-1980)

I dedicate this thesis with fond memories to my father who died at a very young age after a long fight with pulmonary hypertension. I am so proud to have known you, and with the little memory I have of you, your unconditional love, patience and compassion shall always be remembered and will be an inspiration to me.

Acknowledgements

The Fuhrer lab and the Brain Research Institute have provided a wonderful environment for scientific research and left me with fond memories that would always be cherished. Firstly, I would like to thank Prof. Christian Fuhrer for being a source of inspiration all through my scientific pursuit with him. I admire him for his in-depth understanding of science and the clarity with which he communicated during our scientific discussions. His perfect gentlemanliness and fairness towards his students provided an excellent working environment which was cordial and non-competitive, which I think is most essential for any creative activity. To you Christian, I owe my gratitude for all the support and co-operation you have shown during my PhD work.

The Fuhrer lab was a fun environment to work in. My current co-workers Raffaella, Alain, Thomas, Andy, Lena and Dubravka are a great bunch of people. You have made me feel very comfortable in the lab and always helped in times of need. I enjoyed my collaborations with you Raffa and it's a pleasure working with you. I admire you for the selflessness you displayed in helping and in taking care of things in the lab. Thanks for everything and for making the lab such a comfortable place to be in. I have had wonderful scientific discussions with my batch-mates, Alain and Andy. Their constructive criticisms and scientific input during our lab meetings always provided a stimulating working environment. I wish you guys the best. Alain has been my good friend and I shall always cherish the wonderful times and the never ending discussions we've had together. Among my seniors, I would like to thank Angelo, Peggy and Martijn for their ready help and support they gave me during the initial stages of my PhD. Susanne was so much of a support in teaching me cell culture work. Thanks for the patience you displayed in training me with the difficult cell lines. It was fun knowing Kristin too. I enjoyed discussing my work with her and she has always been a source of encouragement.

The Brain Research Institute as such provided a very energetic scientific environment encouraging inter-lab discussions and collaborations within the home. I would particularly like to thank Dr. Matthias Gesemann for his smart molecular biology input and critical assessments during the progress reports. Thanks to Dr. Michaela Thallmair for training me with the cryostat. My collaboration with Prof. Markus Rüegg's lab in Basel with Dr. Shuo Lin was so productive. He was always cheerful and ready to help. I enjoyed my collaborations with him. I would like to thank Prisca and Silvia for their secretarial work. They have been so cooperative and always helped me with the visa arrangements and German translations most cheerfully and willingly. I would also like to thank the service staff of Hifo, especially Eva, Hellmi, Patrick, Rolland and Stefan for their technical support during my PhD.

During my stay in Zurich, I made such wonderful friends who shared a lot of my interests especially in music. We co-founded a team "Taal" and "Geethavani" and it was a lot of fun playing music with you guys. It really lightened my mood and also satisfied the aristic self in me. Roy, Akila, Vageesan, Aloysius, Ravi, Deb, Murali, Uma, Chidu, Priyanka, Anurag, and all you other guys I owe my thanks for your friendship.

To my own love, Karthik, I could have never achieved all this without you. Thanks to you my sweetheart for coming with me to Switzerland and being of immense support and strength. You have inspired me in all ways, your attitude towards life, sensitivity towards science and music has left me with strong admiration for you. I thank you Amma for all the strength and courage you have imbibed in me. To you I owe all that I might have achieved today.

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Abbreviations

α-BT	α-bungarotoxin
α-DG	α-dystroglycan
α-DB	α-dystrobrevin
β-DG	β-dystroglycan
Abl	Abelson tyrosine kinase
ACh	acetylcholine
AChE	acetylcholine esterase
AChR	acetylcholine receptor
AMPA	alpha-amino-3-hydroxyl-5-methyl-4-isoxazolepropionic acid
APC	adenomatous polyposis coli
ARIA	acetylcholine receptor inducing activity
BotA	botulinum toxin A
CNS	central nervous system
D/UGC	dystrophin/utrophin glycoprotein complex
Dvl1	dishevelled-1
DKOs	double knockouts
EPSC	excitatory postsynaptic current
FITC	fluorescein isothiocyanate
F-actin	filamentous actin
G-actin	globular actin
GABA	γ-aminobutyric acid
GFP	green fluorescent protein
GTP	guanosine triphosphate
GPI	glycosylphosphatidylinositol
GDNF	glial-cell-derived neurotrophic factor
LTP	long-term potentiation
MASC	myotube-associated specific component
MuSK	muscle specific kinase
MβCD	methyl-β-cyclodextrin

NMDAR	N-methyl-D-aspartate receptor
NMJ	neuromuscular junction
nNOS	neuronal nitric oxide synthase
NRGs	neuregulins
nAChR	nicotinic acetylcholine receptor
PFA	paraformaldehyde
PSD	postsynaptic density
PTKs	protein tyrosine kinases
PTPs	protein tyrosine phosphotases
Rapsyn	receptor associated protein at synapses
RATL	rapsyn-associated transmembrane linking molecule
RT	room temperature
RTK	receptor tyrosine kinase
SFKs	Src-family kinases
SNc	substantia nigra pars compacta
Tox-P	precipitation using α-bungarotoxin
UGC	utrophin glycoprotein complex
VTA	ventral tegmental area
WASp	Wiskott-Aldrich syndrome

Summary

Synaptogenesis is a key process for the development and functioning of the nervous system. Studies from the neuromuscular junction (NMJ) have contributed much to our understanding of synaptogenesis, due to its relatively easy accessibility and similarities to synapses in the central nervous system (CNS). The NMJ develops in a series of steps first involving formation of high density accumulations of acetylcholine receptors (AChRs) and associated proteins in the postsynaptic membrane underneath the active zones of nerve terminals to initiate further signaling at nascent synapses. Following this, NMJs mature and stabilize for effective signaling which forms the basis for nerve-evoked muscle contractibility. The signaling pathways that mediate early aspects of NMJ formation and those involving postnatal stability are not necessarily the same. Therefore elucidating the molecular pathways and players regulating formation vs. stability of NMJs appears important to understand synaptogenesis in general and may contribute to treatments of several muscle related disorders.

Much is known about the molecular mechanisms that first form NMJs. The neural form of agrin, by activating the muscle-specific receptor tyrosine kinase (MuSK), triggers downstream signaling events that eventually lead to the clustering of AChRs. Within this pathway rapsyn is a crucial protein, transducing tyrosine kinase signaling between MuSK and the AChR. Rapsyn also links the receptor to β-dystroglycan, a member of the dystrophin/utrophin glycoprotein complex (D/UGC). The proteins of the D/UGC complex bridge the extracellular matrix to the internal cytoskeleton thereby stabilizing the postsynaptic membrane. In clustering, AChRs are immobilized and become less detergent extractable. The cytoskeletal intermediates in this pathway still remain unclear. Src-family kinases (SFKs) are known to phosphorylate MuSK and AChRs early in the agrin-induced signaling pathway followed by the kinase Abl, which phosphorylates with different kinetics.

All these players described above are involved in the formation of AChR clusters. The mechanisms for postnatal stability are still poorly understood. Recent studies

have shown MuSK to also have role in stability of NMJs. In addition, in mice lacking utrophin and dystrophin or α-dystrobrevin or dystroglcan (proteins of the D/UGC complex), NMJs form but fail to mature properly. Similarly, studies on SFKs were performed by inactivating the Src and Fyn genes. The neuromuscular junction of mice lacking Src and Fyn were normal around birth and agrin induced normal clustering of AChRs and AChR β subunit phosphorylation in cultured *src*$^{-/-}$*;fyn*$^{-/-}$ myotubes. However when agrin was removed from these cultures, AChRs dispersed rapidly suggesting that SFKs are dispensable during initial stages of AChR clustering but are necessary during later stages of development such as stabilization.

In order to investigate the mechanisms by which SFKs stabilize the NMJ we resorted to in vivo and in vitro methods. Our studies suggest that balanced SFK activity is required for the maintenance of the NMJ. In vivo electroporation of mutant Src constructs into the soleus muscle of adult mice leads to pronounced changes in nerve-muscle topology, synaptic nuclei positioning and severe fragmentation of AChR clusters. This is most likely the result of reduced AChR-protein interactions (e.g. with rapsyn) and AChR β phosphorylation as suggested from immunocytochemical and biochemical studies using *src*$^{-/-}$*;fyn*$^{-/-}$ myotubes. Also, α-tubulin rings underlying the postsynaptic membrane are severely disorganized upon dominant-negative Src electroporation in vivo. All these changes reflect defective cytoskeletal networks and AChR-protein interactions. Consistently, in *src*$^{-/-}$*;fyn*$^{-/-}$ myotubes, the basal cytoskeletal link of the AChRs and the phosphorylation content of key cytoskeletal intermediates such as cortactin and p190RhoGAP are reduced. In addition we find that SFKs are also important in regulating the global levels of rapsyn and cholesterol content in lipid rafts. Lipids rafts are enriched in cholesterol and spingolipids and provide platforms for efficient SFK mediated signaling. Depletion of cholesterol from lipid rafts in wild-type myotubes causes dispersion of AChR clusters, reduced AChR-rapsyn interaction and reduced AChR β phosphorylation. These observations are very much reminiscent of the situation in *src*$^{-/-}$*;fyn*$^{-/-}$ myotubes. Furthermore, addition of cholesterol to the *src*$^{-/-}$*;fyn*$^{-/-}$ myotubes rescues the AChR-instability phenotype seen in these cultures. At the NMJ cholesterol addition promotes the maturation of endplates from patch to pretzel-type configurations in vivo. Taken

together these results suggest a dual mechanism in stabilizing the postsynaptic apparatus. SFKs maintain raft integrity by balanced cholesterol levels and recruiting proteins into rafts; and rafts provide a microenvironment for efficient SFK signaling allowing interactions and phosphorylation of postsynaptic proteins (such as AChR) through rafts.

Together, the data elucidate the mechanism of SFK-mediated postnatal NMJ stability. Multiple pathways seem to converge and act through SFKs in stabilizing the neuromuscular junction. Although SFKs appear passive during initial stages of development, their presence is indispensable in regulating complex signaling pathways that underlie postnatal stability of the NMJ.

Zusammenfassung

Synaptogenese ist ein wichtiger Prozess für die Entwicklung und Funktion des Nervensystems. Studien über die neuromuskuläre Synapse haben wegen deren Zugänglichkeit und Ähnlichkeit zu Synapsen im zentralen Nervensystem entscheidend zum allgemeinen Wissen über Synapsenbildung beigetragen. Die neuromuskuläre Endplatte entwickelt sich in einer typischen Abfolge, die zunächst die Entstehung dichter Ansammlungen von Acetylcholin-Rezeptoren (AChRen) unterhalb der aktiven Zonen der Nervenendigung beinhaltet. Danach reift die Synapse und wird stabilisiert, um effiziente Signalübertragung zu ermöglichen, was eine Grundlage zur Nerv-evozierten Muskelkontraktion bildet. Die Signalmechanismen zur ersten Bildung der Endplatte sind nicht notwendigerweise die gleichen wie zur späteren Stabilisierung. Daher erscheint es wichtig, die molekularen Vorgänge aufzudecken, welche Bildung resp. Stabilitär der neuromuskulären Synapse steuern; die Erkenntnisse werden zum allgemeinen Wissen über Synaptogenese beitragen und auch die Erforschung möglicher Therapien für neuromuskuläre Erkrankungen begünstigen.

Vieles ist schon bekannt über die molekularen Prozesse zur ersten Bildung der Endplatte. Neurales Agrin löst durch Aktivierung der Muskel-spezifischen Kinase (MuSK) weitere Schritte aus, die zur Akkumulation von AChRen führen. Darin ist Rapsyn ein zentrales Protein und leitet Tyrosinkinasen-Aktivität zwischen MuSK und AChRen weiter. Rapsyn verbindet auch den AChR mit β-Dystroglycan, einem Mitglied des Dystrophin/Utrophin-Glykoprotein-Komplexes (D/UGC). Die Bestandteile des D/UGC bilden eine Brücke zwischen der extrazellulären Matrix und dem internen Zytoskelett und stabilisieren damit die postsynaptische Membran. Während ihrer Akkumulation werden AChRen immobilisiert und weniger löslich in Detergenzien. Die Zwischenschritte des Zytoskeletts in diesem Vorgang sind immer noch unbekannt. Tyrosinkinasen der Src-Familie (SFK) können MuSK und AChR früh nach Agrin-Stimulation phosphorylieren und werden später durch Abl-Kinasen abgelöst, die andere Kinetik zeigen.

All diese Mechanismen sind an der Bildung von AChR-Akkumulationen beteiligt. Die Vorgänge der postnatalen Stabilisierung hingegen sind kaum bekannt. Neue Daten zeigen eine Rolle von MuSK auch in diesem Prozess. Zudem bilden Mäuse, denen Utrophin und Dystrophin oder α-Dystrobrevin oder Dystroglycan (Teile des D/UGC-Komplexes) fehlen, normale Endplatten, aber können diese nicht reifen lassen. In ähnlicher Weise wurden Studien mit Mäusen durchgeführt, denen Src und Fyn fehlen. Die neuromuskulären Synapsen dieser Tiere waren normal bei Geburt, und Agrin induzierte normale Akkumulation der AChRen und Phosphorylierung der β Untereinheiten des AChR in *src*$^{-/-}$*;fyn*$^{-/-}$ Muskelzellen. Aber nach Wegnahme von Agrin aus dem Kulturmedium lösten sich AChR-Aggregate schnell auf; daher könnten Src-Familien-Kinasen (SFKen) entbehrlich sein für die ersten Phasen der AChR-Aggregation aber notwendig für spätere Aspekte wie Stabilisierung.

Um die Mechanismen abzuklären, wie SFKen die Endplatte aufrecht erhalten, haben wir in vivo- und in vitro-Methoden verwendet. Unsere Resultate zeigen, dass ausbalancierte Aktivität der SFKen nötig ist zur Stabilisierung der neuromuskulären Synapse. In vivo-Elektroporation mutanter Src-Konstrukte in den Soleus-Muskel erwachsener Mäuse rief ausgeprägte Änderungen in der Nerv-Muskel-Topologie und der Anordnung synaptischer Muskelzellkerne und schwere Fragmentierung der AChR-Aggregate in der postsynaptischen Membran hervor. Dies ist eine Folge von reduzierten AChR-Protein-Wechselwirkungen (z.B. mit Rapsyn) und β Phosphorylierungen, wie unsere immunocytochemischen und biochemischen Studien an *src*$^{-/-}$*;fyn*$^{-/-}$ Muskelzellen vermuten lassen. Zudem war nach Elektroporation mutanter, dominant-negativer Src-Konstrukte in vivo die subsynaptische Anordnung von α-Tubulin in Ringen gestört. All diese Abnormalitäten widerspiegeln Defekte im Zytoskelett und in AChR-Protein-Interaktionen. Dementsprechend waren die Bindung des AChRs ans Zytoskelett und der Phosphorylierungszustand von wichtigen Zytoskelett-Modulatoren wie Cortactin und p190RhoGAP abgeschwächt in *src*$^{-/-}$*;fyn*$^{-/-}$ Muskelzellen. Wir fanden heraus, dass SFKen auch den Protein-Spiegel von Rapsyn und den Gehalt an Cholesterin in Lipid-Mikrodomänen (sog. Rafts) steuern. Lipid-Rafts sind angereichert in Cholesterin und Shingolipiden und stellen Plattformen für effiziente SFK-Signalisierung dar. Wegnahme von Cholesterin aus

Lipid Rafts in normalen Muskelzellen führte zur Auflösung von AChR-Aggregaten, reduzierten AChR-Rapsyn-Interaktionen und AChR β Phosphorylierung – genau so, wie in *src*$^{-/-}$;*fyn*$^{-/-}$ Zellen ohne Cholesterin-Wegnahme. Zugabe von Cholesterin zu *src*$^{-/-}$;*fyn*$^{-/-}$ Zellen korrigierte die Unstabilität der AChR-Aggregate. In vivo-Zugabe von Cholesterin begünstigte die Reifung der Endplatten. All diese Resultate legen eine dualen Mechanismus nahe in der Stabilisierung der Endplatte. SFKen erhalten die Lipid Rafts durch ausbalancierten Cholesterin-Spiegel und durch Rekrutierung postsynaptischer Proteine in Rafts; Rafts wiederum stellen eine Mikro-Umgebung zur Verfügung, in der SFKen Wechselwirkungen und Phosphorylierungen postsynaptischer Proteine auslösen können.

Zusammenfassend decken diese Daten den Vorgang der SFK-vemittelten postnatalen Stabilisierung der neuromuskulären Endplatte auf. Verschiedene Prozesse konvergieren und geschehen durch SFKen in dieser Entwicklungsphase. Obwohl die Kinasen passiv erscheinen in den ersten Stadien der Endplattenentwicklung, spielen sie eine grosse Rolle in der Regulation komplexer Signalisierungsnetzwerke, die postnatale Stabilität der postsynaptischen Membran garantieren.

Chapter 1

Introduction

Synapses are essential relay stations for the transmission of information between neurons and other cells. Rapid and precise synaptic transmission requires a high concentration of neurotransmitter-filled synaptic vesicles in the presynaptic nerve terminal. The vesicles are apposed by highly concentrated neurotransmitter receptors in the postsynaptic membrane. Concentration of these receptors in the postsynaptic apparatus in clusters is thus an essential aspect of chemical synapses. Neurotransmitter receptor clustering at the postsynapse is spatially and temporally regulated. The best-characterized synapse in this respect is the vertebrate neuromuscular junction (NMJ). Due to its easy accessibility and manipulation both in vivo and vitro, and based on the many structural plus functional similarities with the synapses in the central nervous system, the NMJ provides a good model system for synapse formation in general. It provides basic information about synapse formation, maintenance and elimination, which may also apply to other synapses.

Components of the NMJ

The NMJ comprises three cell types - the motor neuron, muscle fibre and Schwann cell (Figure 1). The motor nerve terminal is specialized for neurotransmitter release. It contains 50 nm diameter synaptic vesicles filled with the neurotransmitter acetylcholine, as well as numerous mitochondria that provide energy for the synthesis and release of transmitter. Many vesicles are focused as dense patches at a portion of the presynaptic membrane called active zone, where vesicles fuse and release their contents into the synaptic cleft. The muscle fibre, at the postsynaptic specialization of the NMJ, carries a high concentration of acetylcholine receptors (AChRs) to respond rapidly and reliably to neurotransmitter release. The postsynaptic membrane of the muscle fibre forms invaginations into approximately 1 μm-deep junctional folds that directly appose the active zone of the nerve terminal, an arrangement that enlarges the postsynaptic surface, and may thus enhance the efficacy of synaptic transmission. The AChRs are concentrated at the crests and partly down the sides of the folds. The

Schwann-cell processes cap the nerve terminal, insulating it from the environment and providing trophic sustenance.

Finally, the basal lamina ensheathes the muscle fibre, passing through the synaptic cleft and extending into the junctional folds at the NMJ, where it contains specialized synaptic components like collagen IV, laminin forms, entactin, acetylcholinesterase, neuregulin and heparin sulphate proteoglycans like agrin.

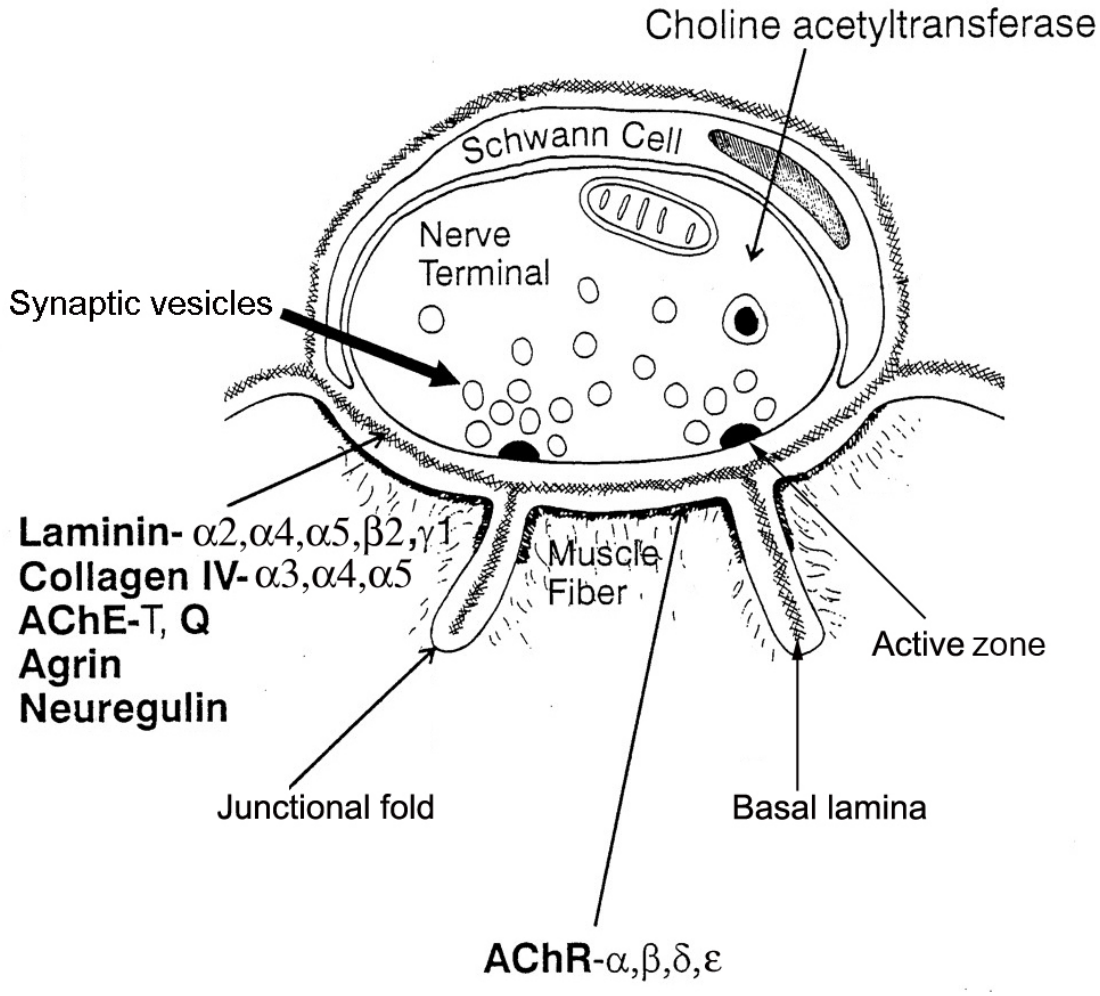

Figure 1: Schematic cross section through an adult neuromuscular junction showing that active zones in the nerve terminal directly appose junctional folds in the postsynaptic membrane. Proteins present in the synaptic basal lamina and the AChRs in the postsynaptic apparatus are indicated (adapted from Sanes and Lichtman, 1999).

Formation of the NMJ

In vertebrates the outgoing motor nerve contacts the muscle late during embryonic development, when myoblasts differentiate into myotubes. Over a period of about a week, fully functional synapses form, in which the nerve and muscle are greatly transformed indicating that several signals are exchanged to initiate an assembly of highly specialised pre- and postsynaptic apparatus (Figure 2). Studies based on damaging the nerve or the muscle, followed by regeneration, indicated that the signals which cause postsynaptic differentiation are stably located in the synaptic basal lamina (Anderson and Cohen, 1977; Burden et al., 1979; McMahan et al., 1980). There is some evidence that this basal lamina also contains signals that direct presynaptic differentiation, but little is known about the nature of these signals.

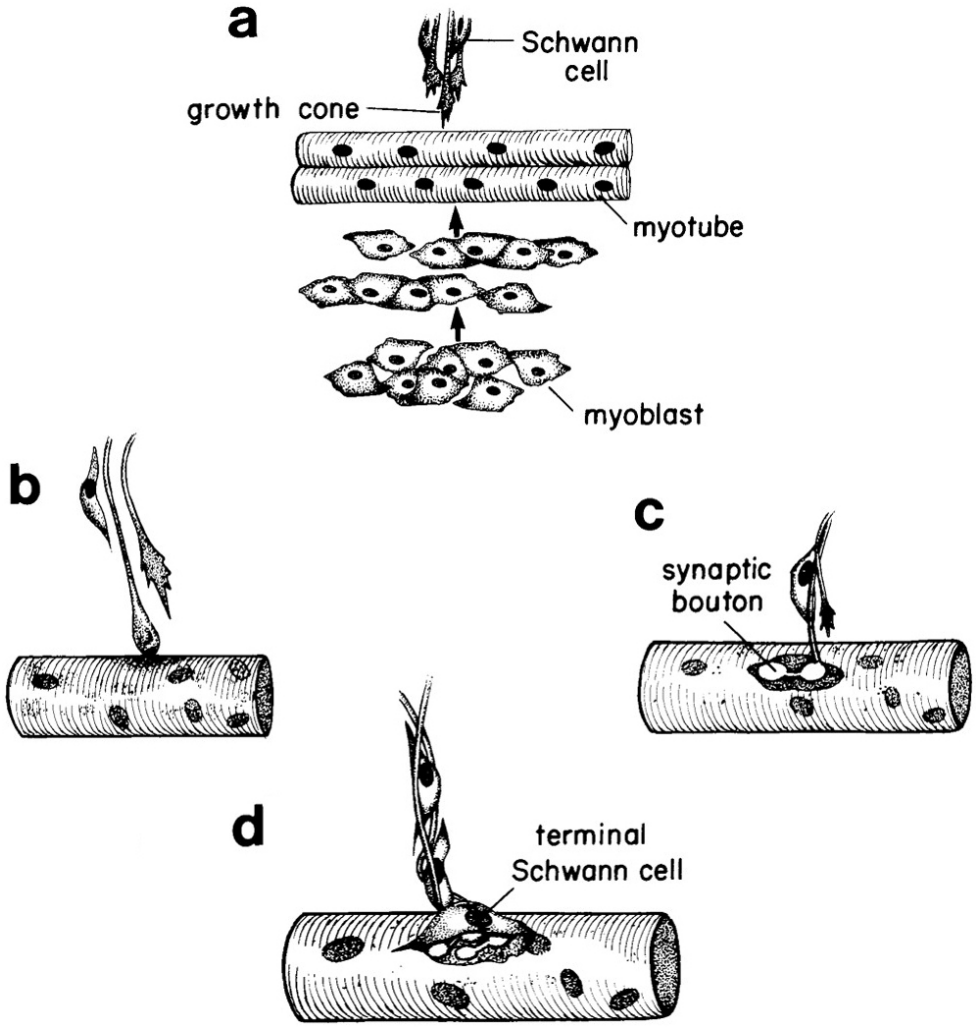

Figure 2: Early steps involved in the formation of the neuromuscular junction. (a) Myoblasts fuse to form myotubes. During differentiation they are approached by motor axons and Schwann cells. (b) Initial contact of the nerve and the muscle. (c) Growth cones differentiate into nerve terminals, and AChRs accumulate in the postsynaptic membrane. (d) By birth the NMJ is fully functional and multiply innervated. In the first weeks after birth of a rodent, all but one axon are eliminated, resulting in a pattern where each muscle fiber receives input from only one motor neuron (adapted from Sanes and Lichtman, 1999).

Postsynaptic differentiation

Myoblasts align and fuse to form myotubes, upon which many contractile and synaptic proteins are activated. Motor axons exit from the central nervous system through ventral roots or cranial nerves, then run long distances through peripheral nerves to muscles. A single motor nerve innervates a single muscle and branches intramuscularly to innervate tens to hundreds of muscle fibres. Motor axons reach the target muscles as myoblasts fuse to form myotubes. Once the motor axon's growth cone contacts a newly formed myotube, synaptic transmission commences quickly. AChRs are initially expressed at low levels in myoblasts and then upregulated upon fusion. The AChR subunits are translated, assembled, and inserted into the plasma membrane and reach a uniform density of ~1000/μm^2. Much in contrast, in mature muscles the density of AChRs reaches 10,000/μm^2 synaptically and falls to ~10/μm^2 extrasynaptically. Three distinct processes contribute to this redistribution: clustering of diffusely distributed AChRs in the postsynaptic membrane, transcriptional activation of AChR subunit genes in subsynaptic nuclei, and transcriptional repression of AChR subunit genes in nonsynaptic myonuclei (Sanes and Lichtman, 1999). Initially, AChR clusters are also seen at a distance from the nerve; this transient process, which can occur in the complete absence of the motor neurons, has been termed muscle pre-patterning (Lin et al., 2000; Yang et al., 2001). It indicates that the muscle can achieve a certain degree of postsynaptic differentiation independently of the nerve.

Formation of AChR clusters

There are two main aspects of synaptogenesis with respect to AChR clustering at the NMJ: one is the clustering of the receptor in a flat postsynaptic membrane, which is an early event of postsynaptic differentiation; the other is the maintenance of such high-density receptor clusters at the crests of the forming postjunctional folds, which occurs postnatally. The following section describes the formation of AChR clusters.

The Agrin-MuSK signalling pathway

Agrin, a large heparin sulphate proteoglycan, is a main nerve-derived organizer of postsynaptic differentiation at the NMJ. It is synthesized by the motor neurons and released from the nerve terminals, where it stably associates with the basal lamina of the synaptic cleft. Alternatively spliced forms of agrin known as the z+ agrin expressed only by the neurons are ~1000 times more potent in AChR clustering in vitro as compared to the z- form expressed by the muscle and Schwann cells. Selective genetic disruption of the z+ agrin form is as deleterious to NMJ formation as the null allele. The animals lack differentiated NMJs and clusters of AChRs and other postsynaptic marker proteins in association with the nerve at birth, by which time the mice die due to respiratory failure (Gautam et al., 1996). Chimeric synapses made by agrin-positive neurons on agrin-deficient myotubes are normal (Burgess et al., 1999). Together these results indicate that the nerve derived z+ agrin is crucial for postsynaptic differentiation.

The z+ agrin activates a receptor in the muscle membrane, but the full identity of this receptor is still unclear. One part of the agrin-receptor is MuSK, a transmembrane receptor tyrosine kinase that is selectively activated by z+ agrin (neural agrin). MuSK was originally identified in *Torpedo* electric organ (Jennings et al., 1993) and is highly expressed in myotubes and muscle fibres (Valenzuela et al., 1995). Muscles in MuSK–null mutant mice lack differentiated NMJs and clusters of AChRs and other postsynaptic marker proteins. They show similarities in their phenotype to animals lacking agrin, which further supports the idea that MuSK is a part of the agrin receptor (DeChiara et al., 1996). Furthermore the *MuSK* $^{-/-}$ myotubes are completely unresponsive to agrin in that tyrosine phosphorylation events (such as

phosphorylation of the AChRs) are not initiated and the AChRs not clustered (Glass et al., 1996; Zhou et al., 1999). Gain-of-function tests on the roles of MuSK were done by the introduction of a constitutively active recombinant form of MuSK at ectopic sites in innervated muscles in vivo. Interestingly, at these sites a full complement of postsynaptic proteins accumulated including high density AChR clusters (Jones et al., 1999; Moore et al., 2001). Together these results demonstrate that MuSK acts downstream of agrin and is a part of the functional receptor of agrin. MuSK is both necessary and, upon activation, sufficient to drive postsynaptic assembly.

The complete signalling pathway downstream of MuSK leading to AChR clustering and postsynaptic differentiation is still unclear, but an increasing number of intermediate proteins are being identified. These include the MuSK-binding proteins dishevelled (Luo et al., 2002) and geranylgeranyltransferase (Luo et al., 2002; Luo et al., 2003), the small GTPases Rac, Rho and Cdc42 (Dai et al., 2000; Weston et al., 2000), the tyrosine kinases Abl-1 and Abl-2 (Finn et al., 2003), the tumor suppressor APC (Wang et al., 2003) and the kinase PAK (Luo et al., 2002). The coordination of these intermediates into a coherent signalling cascade downstream of MuSK is currently unclear. It is also unknown how they interact with the key downstream effector in postsynaptic differentiation, the AChR-scaffolding protein rapsyn. Rapsyn is a 43KDa membrane-associated cytoplasmic protein and is present at roughly 1:1 stoichiometry with AChRs at the adult neuromuscular junction and in Torpedo electric organ (LaRochelle and Froehner, 1986, 1987). Both gain and loss of function tests for rapsyn have given consistent results. Co-expression of rapsyn and AChRs in heterologous non-muscle cells leads to the formation of AChR-rapsyn co-clusters, whereas AChRs are diffusely distributed when expressed on their own (Froehner et al., 1990; Phillips, 1995). No AChR clusters form in rapsyn-null mutant mice. Likewise, myotubes isolated from the mutants and treated with a variety of clustering agents, do not form spontaneous or agrin-induced AChR clusters (Gautam et al., 1995). However MuSK is still localized normally at the synaptic sites and is activated by agrin in the *rapsyn* $^{-/-}$ mutant, indicating that rapsyn acts downstream of MuSK (Apel et al., 1997). The level of rapsyn expression is an important parameter that

determines its ability to mediate agrin-induced AChR clustering in myotubes. When rapsyn is overexpressed in myotubes, fewer spontaneous and agrin-induced AChR clusters are formed than in myotubes expressing physiological levels of rapsyn (Yoshihara and Hall, 1993). This implies that in muscle, other muscle proteins may regulate rapsyn aggregation. Moreover, rapsyn is also somehow involved in the transport of AChR to the plasma membrane (Han et al., 2000; Marchand et al., 2000). It is however unclear whether it is a requirement for regulated AChR surface transport and whether it regulates this transport positively or negatively. Rapsyn-AChR interaction is regulated by agrin, as z+ agrin increases this interaction at the muscle surface (Moransard et al., 2003). By many criteria this agrin-induced increased interaction between rapsyn and AChRs correlates with clustering. It occurs with AChRs that are linked to the cytoskeleton. Thus, a possible mode of action of rapsyn is that its increased AChR-interaction induced by agrin somehow regulates cytoskeletal interactions of the AChR. Interestingly, AChRs are required for rapsyn to form synaptic clusters, both in cultured myotubes (Marangi et al., 2001) and in mutant zebrafish (Ono et al., 2001). Rapsyn and AChRs thus seem to by interdependent components of a protein complex that mediates clustering. By these criteria agrin, MuSK, rapsyn and AChRs constitute a core programme of postsynaptic differentiation at the NMJ (Figure 3). The aforementioned signalling intermediates downstream of MuSK are most likely regulators of this core programme.

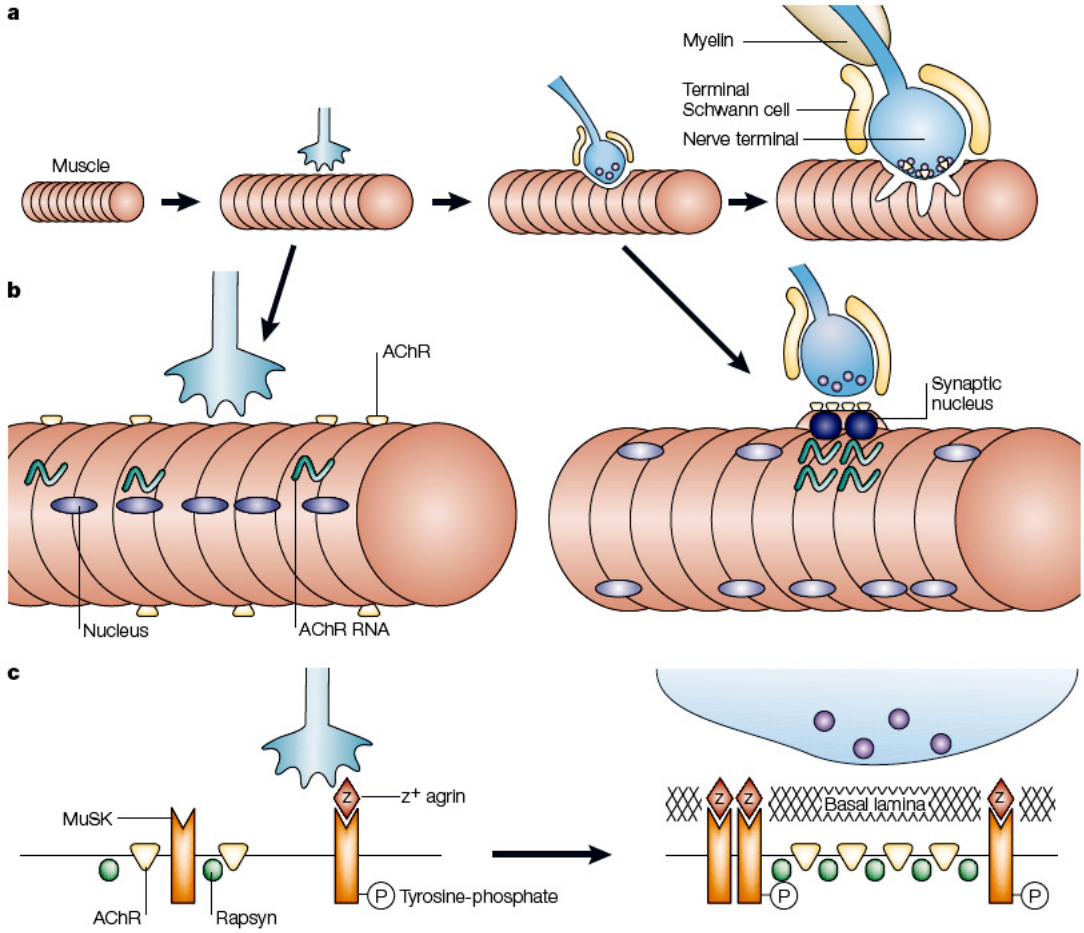

Figure 3: Outline of NMJ formation. a) The motor nerve approaches a newly formed myotube. At the site of contact, the axon differentiates into a motor nerve terminal that is specialized for transmitter release, and the muscle forms a complex postsynaptic apparatus. b) AChRs are initially diffusely distributed on the myotube surface. In adult muscle, AChRs are highly concentrated in the postsynaptic membrane and virtually absent extrasynaptically. This clustering involves both redistribution of AChR proteins, and localized synaptic synthesis of AChRs. c) Neural agrin, the z+ form derived from the presynaptic nerve terminal binds to its co-receptor MuSK, triggering AChR clustering in a rapsyn dependent fashion on the postsynaptic membrane (adapted from Sanes and Lichtman, 2001).

Neuregulin receptors and subsynaptic gene transcription

High density accumulation of synaptic proteins, particularly AChRs, at the NMJ is partly achieved by the activity of neuregulin-1, a protein expressed in motor neurons, terminal Schwann cells and muscle fibers. Motor neuron derived neuregulin-1 is a

member of the neuregulin gene family and was first isolated from chick brain as AChR-inducing activity (ARIA) because of its ability to induce AChR transcription in cultured myotubes (Falls et al., 1993; Fischbach and Rosen, 1997). Neuregulins bind and activate ErbB receptor tyrosine kinases (ErbB2-4), which are concentrated in the postsynaptic membrane (Moscoso et al., 1995) and the receptors stimulate the same regulatory elements as the motor nerve (Chu et al., 1995; Jo et al., 1995; Schaeffer et al., 1998). Thus nerve derived neuregulin appeared to promote local transcription of AChRs. Genetic analysis of neuregulin proved difficult as mutants lacking neuregulin or ErbBs died early in embryogenesis due to cardiac defects (Morris et al., 1999; Woldeyesus et al., 1999). Transgene rescue experiments or muscle-specific ablation of both ErbB2 and ErbB4 have been used to circumvent embryonic lethality (Escher et al., 2005). This rendered muscle cells insensitive to neuregulin while preserving signalling to Schwann cells and cardiac muscle, which is critical for survival. The resulting animals were viable, possessed normal strength and had structurally and functionally normal NMJ in many respects, although the AChR density was slightly reduced by 20-30% in subsynaptic nuclei. Taken together, these results demonstrate that neuregulin signalling to muscle is dispensable for at least principle aspects of postsynaptic development.

Crosstalk: neuregulin and agrin signalling pathways

If neuregulin signalling is not required for NMJ formation, the question arises how the neural induction of synapse-specific AChR transcription is mediated. In wild-type muscle, recombinant neural agrin can induce postsynaptic structures, a process depending on AChR gene transcription. Interestingly, ErbB-deficient fibres also formed normal ectopic clusters like the wild-type on neural agrin induction. However the ectopic AChR densities were on an average lower than the nerve induced endplate on the same muscle. Taken together, overexpressed agrin alone can account for almost normal subsynaptic AChR density (Hashemolhosseini et al., 2000). Thus the nerve regulates synapse-specific gene transcription at least for the major part by agrin/MuSK signalling, and the neuromuscular neuregulin signalling cascade is redundant with that of agrin (but not vice versa).

Neurotransmission vs aneural AChR clusters

Despite the impressive evidence suggesting nerve-derived regulation of AChR clustering, several observations called it into question. The following experiments suggested that postsynaptic development can occur in the absence of the nerve. First, muscles that have been genetically rendered aneural formed AChR aggregates, persistent and grouped in central endplate bands (Lin et al., 2001; Yang et al., 2001; Pun et al., 2002). Second, postsynaptic sites were transiently present in agrin mutants although they disappeared before birth. Third, synaptic nuclei beneath the AChR clusters in aneural muscles were transcriptionally specialized casting doubt that the specialization is a consequence of innervation (Lin et al., 2001; Yang et al., 2001). Fourth, rescue of neuregulin or ErbB mutants from cardiac defects or selective deletion of neuregulin isoforms rendered mice with surprisingly mild postsynaptic defects (Morris et al., 1999; Woldeyesus et al., 1999; Wolpowitz et al., 2000). So the the question arises regarding the role of pre-existing clusters, neurotransmission, agrin, neuregulin and synaptic nuclei. One possibility is that pre-patterned sites, like spontaneous clusters in vitro, are dispersed by the nerve (Anderson and Cohen, 1977; Frank and Fischbach, 1979). Alternatively pre-pattered sites may form as a result of failed innervation. Or they are recognized and incorporated into synapses. Recent studies in zebrafish embryos revealed that postsynaptic AChR aggregates formed in advance of nerve innervations. Some aggregates formed even before the nerves left the spinal chord or where the axon extension was prevented. Although some of them dispersed before innervation others were incorporated into synapses. Axons were however required for maintenance of postsynaptic sites as prevention of axon outgrowth resulted in eventual loss of AChR aggregates (Brandon et al., 2003; Flanagan-Steet et al., 2005). Thus pre-patterned postsynaptic specializations can participate in normal synaptogenesis, although their maintenance requires innervation.

To specifically address how neurotransmission affects early aspects of synaptogenesis, ChAT (choline acetyltransferase) mutant mice were generated (Misgeld et al., 2002; Brandon et al., 2003). ChAT is a sole synthetic enzyme for producing ACh, and ChAT mutant NMJs revealed numerous defects in early postsynaptic differentiation. Early nerve branching patterns were abnormal and each

myotube bore more than one NMJ. In addition the endplate band and their associated AChR clusters with specialized nuclei were widened in the absence of synaptic transmission. Interestingly, the cross between knockouts of agrin and ChAT (resulting in double-knockout) remarkably formed NMJs, underwent pre- and postsynaptic differentiation and persisted until birth. AChR density and cluster size were normal, although atrophy was severe and some fibres were uninnervated. These results imply that the primary function of agrin is to counteract the local dispersal effects of neurotransmission at synaptic sites. In vitro analysis supported this idea, as charbacol (cholinergic agonist) dispersed spontaneous AChR clusters; but dispersal was blocked on sites of contact with heterologous cells that expressed z+ agrin compared to z– agrin (Bloch, 1986; Misgeld et al., 2002; Lin et al., 2005).

These data suggest that agrin counteracts activity-induced dispersal, functioning at least in part as an anti-declustering factor to stabilize nerve-contacted AChR aggregates.

Tyrosine kinase signalling in AChR clustering

Phosphorylation of the AChR β subunit

Another key aspect of the agrin-induced MuSK signalling pathway is the phosphorylation of the AChR on tyrosine residues. Tyrosine phosphorylation of AChRs occurs on both β and δ subunits in cultured myotubes and is paralleled by the link of the receptor to the cytoskeleton (Wallace, 1992; Ferns et al., 1996; Mittaud et al., 2001; Mittaud et al., 2004). Similarly, in vivo the AChRs are progressively stabilized, most likely through some sort of cytoskeletal link whose nature is not entirely clear, and these junctions contain phosphotyrosine proteins that co-distribute with the receptors (Froehner et al., 1990; Meier et al., 1995). In order to determine if the agrin-induced clustering and cytoskeletal link of the AChR requires tyrosine phosphorylation several experiments were done. For example, tyrosine kinase inhibitors like herbimycin and staurosporin blocked agrin-induced clustering and phosphorylation of the AChR (Wallace, 1995; Ferns et al., 1996; Mittaud et al., 2004). Pervanadate, an inhibitor of tyrosine phosphatases, caused increased phosphorylation and cytoskeletal link of the receptor in myotubes (Wallace, 1995).

Most important, transfection of myotubes with mutant AChRs lacking cytoplasmic tyrosine residues in their β subunit showed that although some AChRs were still able to form agrin-induced clusters (Meier et al., 1998), the extent of clustering and cytoskeletal linkage was clearly reduced (Borges and Ferns, 2001). This demonstrates that tyrosine phosphorylation of the AChR β subunit induced by agrin regulates the link of the receptor to the cytoskeleton and contributes to efficient clustering of the receptor.

The question then arises as to what kinases are involved in the phosphorylation of the AChRs. Accumulating evidence suggests that Src-family kinases are associated with both MuSK and the AChR in myotubes and are activated by agrin. Src and Fyn associate with the AChRs in myotubes (Fuhrer and Hall, 1996) and are involved in early aspects of receptor phosphorylation, i.e. in very early phases after addition of agrin (Mohamed et al., 2001; Smith et al., 2001; Mittaud et al., 2004). A Src kinase inhibitor, PP1, blocks agrin-induced AChR-cytoskeletal link and expression of a dominant-negative kinase-inactive Src in myotubes interferes with AChR clustering leading to receptor aggregates that are very small (Mohamed et al., 2001). However, PP1 is known to inhibit other kinases such as Abl (Tatton et al., 2003; Warmuth et al., 2003), and the dominant-negative Src expression was done in myoblasts and may have affected myotube differentiation. The role of Src-family kinases in the formation of AChR clusters is therefore unclear. This is further illustrated by other studies using PP1 and also a more specific Src inhibitor (CGP77675) – these studies failed to see an effect on AChR cluster formation (Smith et al., 2001; Mittaud et al., 2004). Nonetheless, it is speculated that the phosphorylation of rapsyn and/or α-dystrobrevin-1 by Src-class or other kinases may also be important for formation and maturation of the NMJ (Figure 4).

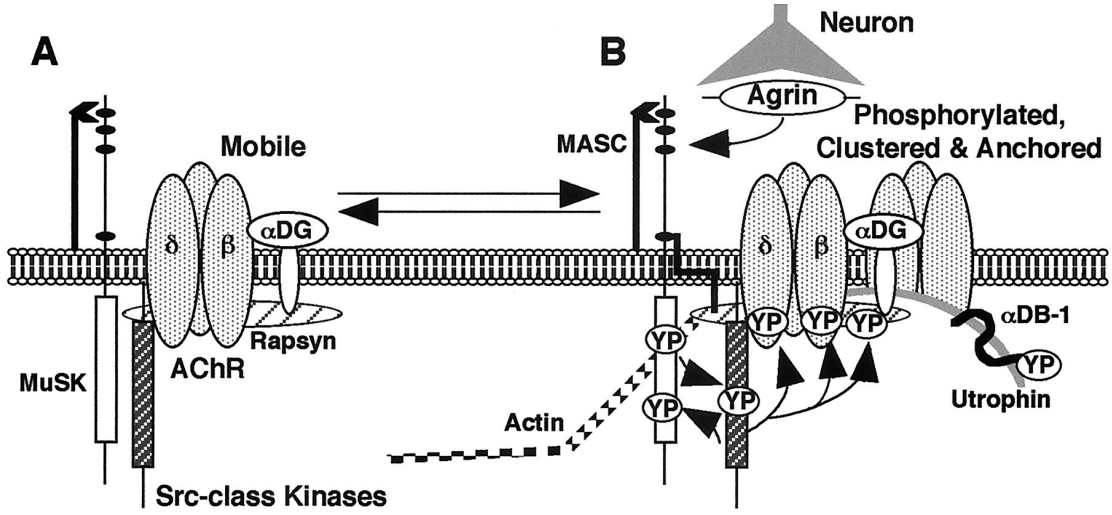

Figure 4: Model for the role of Src-class protein tyrosine kinases in phosphorylation and cytoskeletal anchoring of AChRs at the NMJ (adapted from Mohamed et al., 2001). Abbreviations: α-DG, α-dystroglycan; αDB-1, α-dystrobrevin-1; MASC, myotube-associated specificity component (hypothetical MuSK-interacting coreceptor for agrin).

Structure of Src

The members of the Src-family kinases (SFKs) are divided into two classes: tyrosine kinases with broad expression range (Src, Fyn,Yes) and those with limited expression (Fgr, Lyn, Hck, Lck, Blk, Yrc). In vertebrates the proteins of the Src family have a similar structure. The proteins range in molecular mass from 52 to 62 kD comprising six distinct functional domains: Src homology domain 4 (SH4), a unique domain, SH3 domain, SH2 domain, a catalytic domain (SH1), and a C-terminal regulatory domain (Figure 5).

The N-terminal SH4 domain is a region containing 15-17 amino acids that bear a signal for myristylation and also one for palmitoylation, which directs the association of these proteins to cellular membranes. The following 40-70 amino acids are poorly conserved among the different members. This region, called the unique domain, is suggested to be responsible for specific interactions of the Src-kinases with particular receptors and protein targets. C-terminal to the unique domain is the SH3 domain, a structure of 60 residues capable of protein-protein interactions (Figure 5). It ensures intra- and intermolecular bindings controlling catalytic activity,protein localization in

the cell, and association with protein targets. Next to the SH3 domain is the SH2 domain, comprising about 100 residues. The SH2 domain is a high affinity binding unit for phosphotyrosine–containing proteins and is frequently found in proteins involved in signal transduction. This domain mediates interaction with receptor-type protein tyrosine kinases, cytoskeletal substrates and may also mediate interactions with protein substrates of Src kinases. C-terminal to the SH2 is the calaytic domain. It is responsible for tyrosine kinase activity and plays a crucial role in substrate specificity. Certain amino acid residues within this domain are identical in all kinases and involved in ATP binding and phosphotransferase reaction. Within this domain phosphorylation of Tyr-416 stimulates complete activation of Src and provides a binding site for SH2 domains of other cellular proteins. The elimination of Leu-516, highly conserved in all protein tyrosine kinases, interferes with the transforming activity of p60 v-Src. The C-terminal region (amino acid residues from 517 to ~536) plays a significant role in regulation of Src kinase activity. It has been shown that elimination of a conserved amino acid in all Src family members (Tyr-527) increases kinase activity. Phosphorylation of this C-terminal Tyr inhibits kinase activity and suppresses all stimulating effects caused by phosphorylation of Tyr-416 in the catalytic domain.

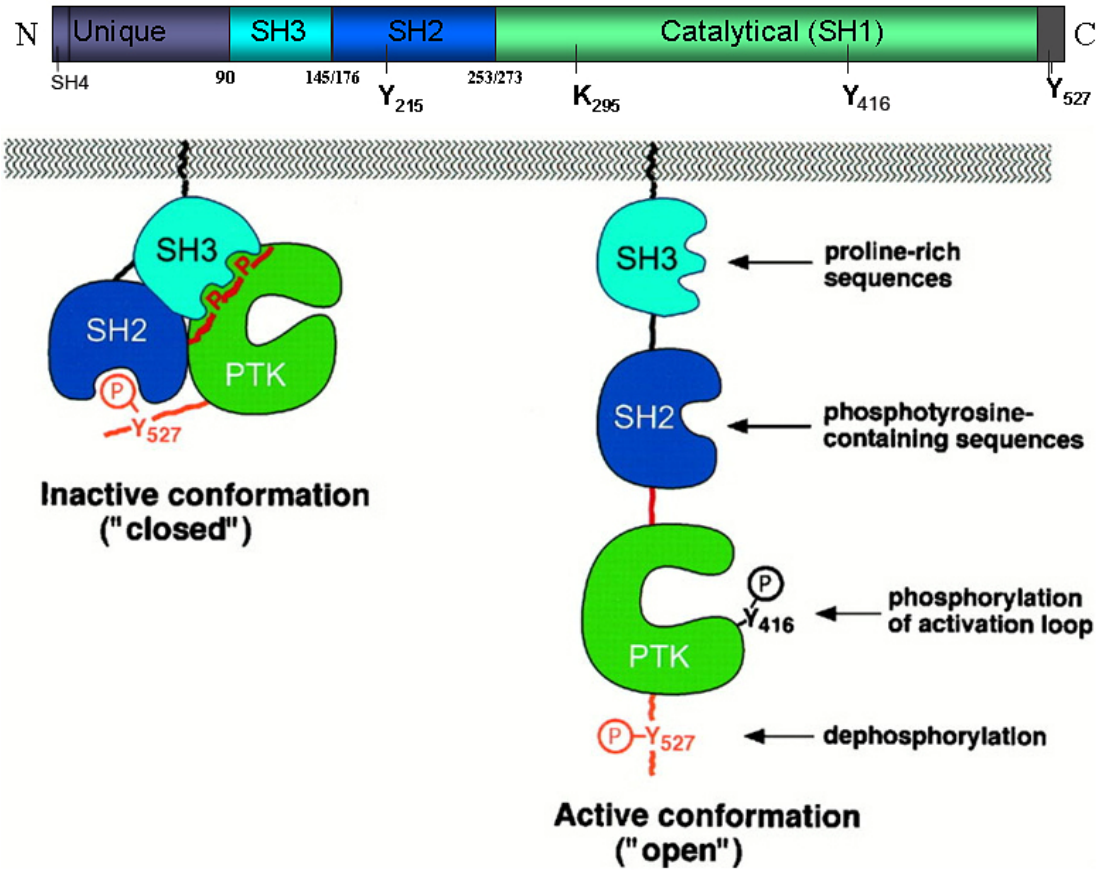

Figure 5: Structure and regulation of Src activity. The closed autoinhibited and open active conformations are induced either by the interactions of the SH2 and SH3 domains with other proteins or by dephosphorylation of the C-terminal Tyr-527. This makes Tyr-416 accessible for phosphorylation (reviewed by Stevan et al., 1998).

Role of SFKs: studies of *src*$^{-/-}$*;fyn*$^{-/-}$ mice

The clearest evidence for the role of the Src-family in postsynaptic differentiation came from genetic studies where mutant mice that lacked both Src and Fyn were generated (Smith et al., 2001). These animals die around birth and move and breathe weakly, suggesting an NMJ failure. However analysis of these animals shows normal synaptic development, such as innervation, AChR gene expression and clustering of postsynaptic proteins. Consistently, in myotube cultures from these mutants, agrin induced normal phosphorylation and clustering of the AChRs. Most interestingly,

after cluster induction, agrin withdrawal from these myotubes resulted in rapid dispersal of AChR clusters. These studies show that Src and Fyn are dispensable for AChR clustering and phosphorylation. Nevertheless, Src and Fyn are required to stabilize agrin-induced AChR clusters, indicating that formations versus stabilization are two independent aspects to synaptogenesis (Smith et al., 2001).

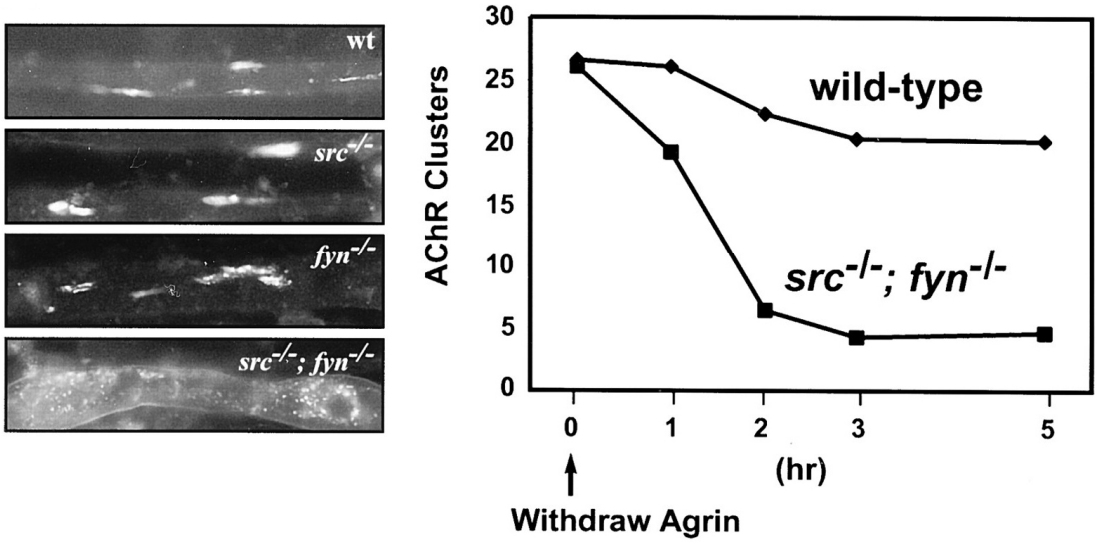

Figure 6: Muscle cells indicating AChR cluster dispersal in Src and Fyn double knockout myotubes after agrin withdrawal. Agrin was added overnight to the myotube cultures and then withdrawn for 3 hours (left) or for the indicated times (right). Clusters were visualized by rhodamine-bungarotoxin staining and counted (adapted from Smith et al., 2001).

Stabilization of AChR clusters

Involvement of the D/UGC complex

Besides tyrosine kinases several components of the dystrophin/utrophin glycoprotein complex (D/UGC) are mainly required for postjunctional fold formation as well as consolidation and stabilization of AChR clusters. They play an important role in linking the muscle cytoskeleton to the extracellular matrix (Straub et al., 1999), thereby stabilizing the sarcolemma. Accordingly, when components of this complex are missing or damaged, this leads to AChR cluster instability and muscular dystrophy (Campbell and Crosbie, 1996; Deconinck et al., 1997; Deconinck et al., 1997; Cote et al., 1999; Grady et al., 1999; Grady et al., 2000). Utrophin and dystrophin, two homologous proteins, are the largest D/UGC components and together are necessary for muscle fibre intergrity but are dispensible for NMJ formation. The single knockouts for dystrophin or utrophin are remarkably healthy and show normal NMJs, except for reduced densities of AChRs and postjunctional folding (Deconinck et al., 1997; Grady et al., 1997). However, a double knockout mice shows severe muscular dystrophy resembling Duchenne muscular dystrophy yet displays NMJs that are relatively normal with a mild phenotype (Deconinck et al., 1997; Grady et al., 1997). A critical component of the D/UGC is dystroglycan, which is important for the consolidation and stabilization of AChR clusters at the NMJ. In order to circumvent the early lethality of *dystroglycan* $^{-/-}$ mice, chimeric mice were generated that lacked dystroglycan in selected striated muscle fibres. The mice developed severe muscular dystrophy in these fibres and the corresponding NMJs were fragmented (Cote et al., 2002). The AChRs were wider in distribution and accordingly the presynaptic nerve terminal occupied an increased area. Agrin-induced AChR clustering was analysed using myotubes generated from *dystroglycan* $^{-/-}$ embryonic stem cells. Agrin still induced substantial clustering of AChRs in these cells, but utrophin, dystrobrevin, laminin chains, perlecan and AChEsterase failed to cluster. As in vivo, the agrin-induced AChR clusters appeared diffuse and actually consisted of several microclusters that dispersed rapidly after agrin withdrawal. Together these data indicate that dystroglycan is required for the condensation and

stabilization of the AChR clusters, shown both in vivo and in vitro (Cote et al., 1999; Grady et al., 2000; Jacobson et al., 2001).

α-dystrobrevin plays a similar role as dystroglycan, it is also necessary for AChR stabilization. Dystrobrevin knockout mice develop muscular dystrophy. Prenatal NMJ formation and development proceed normally in these animals, but after birth the number of postjuctional folds is reduced and they get relatively flattened (Grady et al., 2000). The distribution of AChRs is strongly affected as they are randomly distributed in both crests and troughs. The AChR clusters appear frangmented in older *α-dystrobrevin* $^{-/-}$ animals. Synaptic localization of α, and β2- syntrophin and nNOS is reduced in mutant endplates. Cultured myotubes from these mice show rapid dispersal of AChR clusters after agrin withdrawal (Grady et al., 2000). Thus, dystrobrevin is required for the maintenance and stabilization of AChR clusters for postjunctional fold formation as well as segregation of AChRs between crests and troughs.

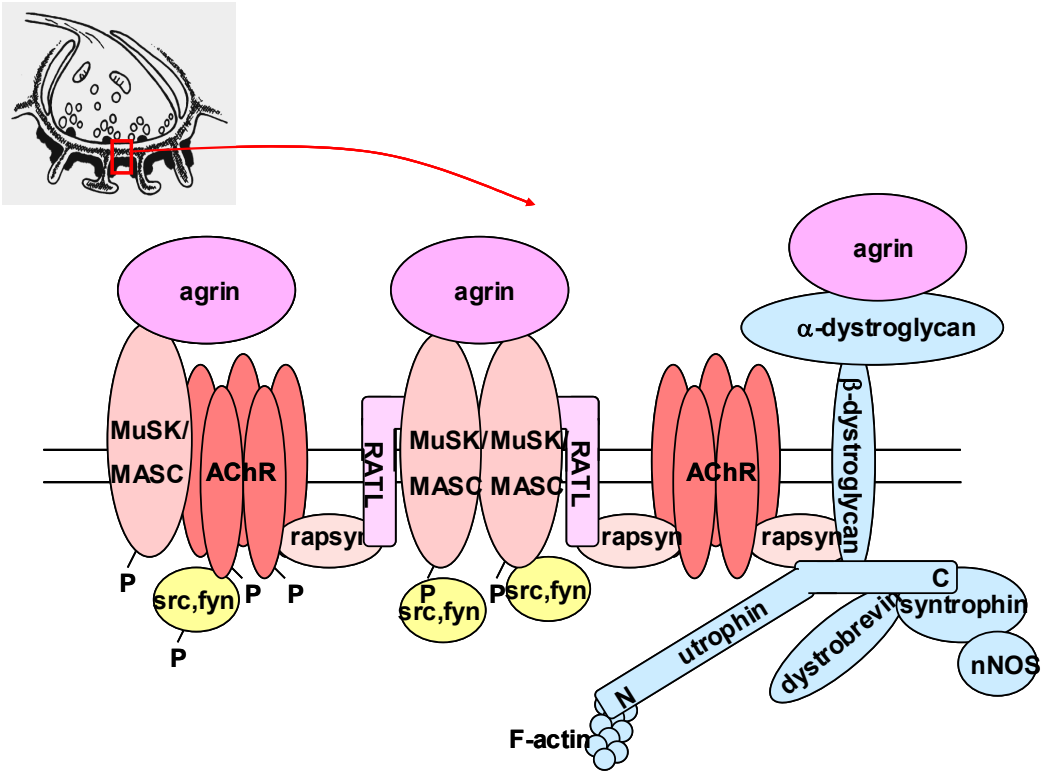

Figure 7: The postsynaptic apparatus at the neuromuscular junction, indicating several proteins that are involved in the formation and stabilization of AChR clusters (adapted from Huh and Fuhrer 2002). The blue coloured proteins together form the dystrophin/utrophin glycoprotein complex (D/UGC).

In summary, AChR clusters are formed by an agrin-induced MuSK signalling pathway, and tyrosine phosphorylation of the AChR β subunit plays a critical role in cluster formation and cytoskeletal anchoring. Once formed, the stability of such clusters requires the presence of Src and Fyn and components of the D/UGC such as dystroglycan and α-dystrobrevin. The pathways for AChR cluster stabilization in myotubes in vitro and for stabilization of the muscle sarcolemma in vivo are similar, as shown in mice deficient for dystroglycan or dystrobrevin, which display both dystrophic muscles in vivo as well as unstable AChR clusters in vitro and in vivo. Therefore, stabilization of AChR clusters can be used as a model system to investigate signalling pathways that maintain the postsynaptic apparatus and that may also be involved in muscle membrane stabilization in general, thereby preventing dystrophy.

Cytoskeletal assembly and AChR clustering

The clustering of AChRs is accompanied by increased anchorage of these receptors to the cytoskeleton and loss of lateral mobility. Studies show that the lateral migration of AChR clusters can be abolished by inhibiting actin polymerization. Inhibitors like latrunculin A, which binds G-actin and prevents the polymerization into F-actin, and Jasplakinolide, another sponge toxin that specifically binds to and stabilizes F-actin, are also shown to block AChR clustering (Dai et al., 2000). These data illustrate that dynamic F-actin assembly is required for AChR clustering. F-actin polymerization is also reported to propel small AChR clusters to the muscle surface at sites of high synaptogenic input and promote their coalescence into larger aggregates, which in turn could be trapped in a corral set up by the actin cytoskeleton, a process referred to as the “diffusion-trap hypothesis” (Poo, 1985).

So the question arises as to what proteins might actually tether the AChR clusters to the cytoskeleton. One candidate could be rapsyn, which is of known importance for AChR clustering. Studies have claimed the association of rapsyn and actin (Walker et al., 1982); although it has remained controversial, this could directly connect rapsyn/AChR complexes to the actin network. Rapsyn also binds to β-dystroglycan of the UGC complex, which in turn contains utrophin that interacts with F-actin as mentioned before (Cartaud et al., 1998; Bartoli et al., 2001). Furthermore a

preliminary report mentioned that rapsyn interacts with a protein that bears high sequence homology to N-RAP, a nebulin related protein expressed in cardiac and skeletal muscle (Tseng et al. 2001- from poster abstract, Society for Neuroscience meeting, San Diego). N-RAP is a modulator protein containing a LIM domain and nebulin repeats and binds actin directly (Luo et al., 1997; Luo et al., 1997).

Importantly, several cytoskeletal intermediates are known to regulate and orchestrate the actin polymerization process in other cell types. Of importance are the proteins of the Arp2/3 complex whose structure and functions have been extensively studied (Pantaloni et al., 2000; Higgs, 2001). But the Arp2/3 complex cannot initiate actin polymerization without activator proteins like WASp, the Wiskott Aldrich Syndrome protein, and its neural isoform N-WASp. The action of WASp on the Arp2/3 complex is positively regulated by small GTPase Cdc42 (Pantaloni et al., 2000). In addition to WASp, cortactin is a protein that activates Arp2/3 and they colocalize at sites of dynamic actin assembly in fibroblasts (Kaksonen et al., 2000). The activity of cortactin on Arp2/3 regulated actin polymerization is in turn regulated by Rac. Interestingly, Rac and Cdc42 have been implied in agrin-induced AChR clustering in muscles and their inhibition is known to completely abolish clustering (Weston et al., 2000). Apart from this cortactin has been shown in Xenopus muscle to cocluster with spontaneous AChR clusters and clusters induced by growth factor-coated beads (Peng et al., 1997; Dai et al., 2000).

Src kinases in cytoskeletal remodelling

An early study of Src-dependent cytoskeletal regulation was accomplished when mutant mice lacking Csk, a negative regulator of Src family kinases were generated. *csk*$^{-/-}$ mice exhibited defects in neurulation and died at mid-gestation (Hamaguchi et al., 1996). Biochemical analysis revealed that many cytoskeletal proteins, particularly cortactin and tensin, were hyperphosphorylated on tyrosine residues. However, crossing *csk*$^{-/-}$ with *src*$^{-/-}$ mice restored normal distribution of cortactin and considerably corrected the filamentous actin organization seen in *csk*$^{-/-}$ mutants (Thomas et al., 1995). Recently several other cytoskeletal intermediates other than cortactin, such as WASp or p190RhoGAP (influencing Rho GTPase activity), have been revealed as substrates of Src-family kinases (Chang et al., 1995; Daly, 2004;

Martinez-Quiles et al., 2004). Their activites on cytoskeletal organization are known to be regulated by an increase or decrease in Src-mediated tyrosine phosphorylation (Thomas et al., 1995; Brandt et al., 2002; Kilarski et al., 2003). An interesting hypothesis would be that the agrin signalling pathway, which activates Src, regulates these cytoskeletal intermediates by tyrosine phosphorylation causing F-actin remodelling and influencing the link and clustering of AChRs.

Lipid rafts as platforms for protein signalling

Lipid rafts are plasma membrane microdomains rich in cholesterol and sphingolipids, providing a particularly ordered environment for cellular signalling. Within these ordered lipid environment, integral and peripheral membrane proteins participate in a regulated fashion, such that a high basal protein concentration and protein-protein interactions can be promoted. The two basic mechanisms by which the lateral dispersion of receptors can be influenced is by the assembly of molecular scaffolds via protein-protein interactions that link the receptor to the cytoskeleton and by the existence of specific domains in the plasma membrane to which the receptors are either inserted or recruited. Thus, lipid rafts could be in a position to concentrate receptors at certain sites, for example neurotransmitter receptors at the synapse.

Several proteins have been identified to be enriched in rafts that are important for growth factor signal transduction, cellular adhesion, axon guidance, vesicular trafficking and synaptic transmission. These include transmembrane and glycosylphosphatidylinositol (GPI)-anchored receptors (Brown and London, 1998; Hooper, 1999), doubly acylated proteins such as Src-family kinases (Resh, 1999), intracellular signalling intermediates such as small GTPases and cholesterol-linked and palmitoylated proteins such as Hedgehog (Rietveld et al., 1999).

Signalling in rafts has been quite extensively studied in immunological synapses in T-cell antigen receptor activation (Janes et al., 2000; Langlet et al., 2000), glial-cell-derived neurotrophic factor (GDNF) signalling (Tansey et al., 2000), for the Ras family of small GTPases (Hancock et al., 1990; Roy et al., 1999) and for Hedgehog signalling (Brdicka et al., 1998; Zhang et al., 1998). Although the exact mechanism of how receptors signal through rafts is still not clear a working hypothesis can be built

based on common observations. Receptors could behave at least in three different ways in rafts. First, receptors associated at steady state with lipid rafts could be activated through ligand binding. Second, individual receptors with weak raft affinity could oligomerize on ligand binding, and this would lead to increased residency time in rafts. Last, activated receptors could recruit crosslinking proteins that bind to proteins in other rafts, and this would result in raft coalescence (Harder et al., 1998; Janes et al., 1999). These models are not mutually exclusive. Through formation of raft clusters, a network of interactions between adaptors , scaffolds and anchoring proteins would be built up to organize the signal complex in space and time. This signalling complex would be insulated within the raft clusters from the non-raft environment such as membrane phosphates that could otherwise affect the signalling process.

One of the useful approaches in raft research has been the manipulation of raft lipid constituents. For instance, depletion of cholesterol by treatment with methyl-β cyclodextrin, leading to raft dispersal, causes loss of surface AMPA receptors and synapses in hippocampal neurons (Hering et al., 2003). In ciliary neurons, α7 neuronal nicotinic acetylcholine receptors are maintained in lipid rafts in synapse-associated clusters (Bruses et al., 2001). At the neuromuscular junction the presence of plasmalemmal cholesterol is necessary for proper gating functions of AChRs (Barrantes, 1993) and in heterologous cells, AChRs associate with lipid rafts in their trafficking to the plasma membrane along microtubule networks (Marchand and Cartaud, 2002).

In the realm of synaptic transmission lipid rafts are emerging as having a structural role, proving a platform for channelling proteins in and out of their selective environment and thus modifying synaptic plasticity and functions.

Goal of this thesis

The NMJ has been used as a model system and extensively studied over decades in several organisms. Most is known about the molecular pathways involved in the formation of NMJs in rodents. Neural agrin, by activating the receptor tyrosine kinase (MuSK), is crucial in triggering a downstream signalling cascade that eventually leads to the phosphorylation and clustering of AChRs in a rapsyn-dependent fashion. Rapsyn plays a key role as an adaptor molecule linking the AChRs to the D/UGC complex which in turn anchors the whole complex to the F-actin cytoskeleton via utrophin. However the molecular players and the mechanisms involved in postsynaptic stabilization are unclear. Recently MuSK has been implicated in stability (Kong *et al*., 2004) besides the proteins of the D/UGC complex as mentioned before. In addition Src-family kinases (SFK) have been shown to be dispensable for the formation of AChR clusters but are crucial for the maintenance of AChR clusters in cultured myotubes.

The goal of this thesis is to investigate whether and by what mechanism, SFKs in particular Src and Fyn, are involved in postnatal stabilization of the NMJ in vivo. For this purpose in vivo studies are combined with experiments using cultured *src*$^{-/-}$*;fyn*$^{-/-}$ myotubes. Since the double knockout mice die early around birth, further analysis of the involvement of Src and Fyn in postnatal NMJ stabilizationcannot be made using these animals. For this purpose another through suitable invivo model system is required. Also, since SFK are ubiquitous and seem to interact with several proteins (MuSK, AChRs) at the NMJ, besides their association with cytoskeletal intermediates and rafts in other cell systems, an elaborate understanding of SFK function and mechanisms in NMJ stabilization is required. Several questions arise as a consequence. How do the SFKs stabilize the postsynaptic apparatus? Do they act by mediating protein-protein interactions or do they regulate signalling by their kinase activity? If so what are the possible SFK substrates at the NMJ and how are they

regulated? How early are SFKs required in postnatal stability, provided that their function is not important in formation of AChR clusters?

To address some of these questions, I have investigated whether SFKs are required to stabilize other postsynaptic proteins along with the AChR clusters in Chapter 2, using in vivo and in vitro models. If so, how do they regulate and maintain interactions with AChRs? Is Src kinase activity important in cluster stability in vivo and vitro? Do SFKs regulate stability by linking protein complexes to the cytoskeleton? In Chapter 3, I aimed at investigating how SFKs interact with and phosphorylate putative downstream cytoskeletal targets in F-actin assembly thereby affecting AChR clustering. Chapter 4 is aimed at investigating how SFKs within lipid rafts regulate postsynaptic development of the NMJ. Finally, in Chapter 5, I discuss in more detail the contribution of these studies to our current understanding of postsynaptic development of the NMJ, and draw possible parallels to synapses in the brain.

Chapter 2

This chapter has been adapted from an article published in The Journal of Neuroscience, Vol. 25, Issue 45, 10479-10493, November 9, 2005 (Sadasivam et al., 2005).

Src-family kinases stabilize the neuromuscular synapse in vivo via protein interactions, phosphorylation, and cytoskeletal linkage of acetylcholine receptors

Abbreviated title: Src action in postsynaptic stabilization

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Acknowledgements: We thank Dr. Mathias Höchli and Dr. Anne Greet Bittermann from the Laboratory of Electron Microscopy at the University of Zürich for their excellent technical assistance with the confocal microscope. This work was supported by the Eric Slack-Gyr Foundation, and by grants from the Swiss National Science Foundation, the Swiss Foundation for Research on Muscle Diseases and the Zürich Neuroscience Center (to C.F.).

Summary

Postnatal stabilization and maturation of the postsynaptic membrane are important for development and function of the neuromuscular junction (NMJ), but the underlying mechanisms remain poorly characterized. We have examined the role of Src-family kinases (SFKs) in vivo. Electroporation of kinase-inactive Src constructs into soleus muscles of adult mice caused NMJ disassembly: acetylcholine receptor (AChR)-rich areas became fragmented, the topology of nerve terminal, AChRs and synaptic nuclei was disturbed, and occasionally nerves started to sprout. Electroporation of kinase-overactive Src produced similar but milder effects. We studied the mechanism of SFK action using cultured *src*$^{-/-}$*;fyn*$^{-/-}$ myotubes, focusing on clustering of postsynaptic proteins, their interaction with AChRs, and on AChR phosphorylation. Rapsyn and the utrophin-glycoprotein complex were recruited normally into AChR-containing clusters by agrin in *src*$^{-/-}$*;fyn*$^{-/-}$ myotubes. But after agrin withdrawal, clusters of these proteins disappeared rapidly in parallel with AChRs, revealing that SFKs are of general importance in postsynaptic stability. At the same time, AChR interaction with rapsyn and dystrobrevin, and AChR phosphorylation decreased after agrin withdrawal from mutant myotubes. Unexpectedly, levels of rapsyn protein were increased in *src*$^{-/-}$*;fyn*$^{-/-}$ myotubes, while rapsyn-cytoskeleton interactions were unaffected. The overall cytoskeletal link of AChRs was weak but still strengthened by agrin in mutant cells, consistent with the normal formation but decreased stability of AChR clusters. These data show that correctly balanced activity of SFKs is critical in maintaining adult NMJs in vivo. SFKs hold the postsynaptic apparatus together through stabilization of AChR-rapsyn interaction and AChR phosphorylation. In addition, SFKs control rapsyn levels and AChR-cytoskeletal linkage.

Introduction

NMJs develop in a series of steps in which the postsynaptic membrane first forms by concentrating AChRs and associated proteins in a flat topology. Postnatally, NMJs mature and AChRs get arranged at the crests of postjunctional folds. Concomitantly, all but one axon withdraw, paralleled by destabilization of adjacent AChRs (Sanes and Lichtman, 2001). Maturation and stabilization of AChR clusters ensure proper synaptic development, which forms the basis for nerve-evoked muscle contractibility.

Much is known about the molecular pathways that first form NMJs. Neural agrin, by activating the kinase MuSK, is crucial by triggering downstream cascades (reviewed by (Bezakova and Ruegg, 2003; Luo et al., 2003). Central in these is rapsyn, the main AChR-anchoring protein mediating clustering (Gautam et al., 1995). Rapsyn increasingly binds to AChRs in response to agrin (Moransard et al., 2003), mediates agrin-induced phosphorylation of the AChR β and δ subunits (Mittaud et al., 2001), and links the receptor to β-dystroglycan, a component of the postsynaptic utrophin-glycoprotein complex (UGC) (Cartaud et al., 1998; Bartoli et al., 2001). In clustering, AChRs become immobilized and less detergent extractable, both in agrin-treated myotubes (Prives et al., 1982; Stya and Axelrod, 1983; Podleski and Salpeter, 1988) and developing NMJs (Dennis, 1981; Slater, 1982). The players in this cytoskeletal link remain uncertain. Agrin-induced phosphorylation of AChR β is involved (Borges and Ferns, 2001) and can occur through Abl and Src-family kinases (Finn et al., 2003; Mittaud et al., 2004).

Much less is known about the mechanisms that mature NMJs and stabilize AChR clusters postnatally. Although MuSK is required (Kong et al., 2004), some of these pathways may not be essential in initial NMJ formation (Willmann and Fuhrer, 2002), as illustrated by mice lacking utrophin and dystrophin or the UGC components α-dystrobrevin or dystroglycan (Grady et al., 1997; Grady et al., 2000; Jacobson et al., 2001). In these mice, NMJs form but fail to mature properly. In *α-dystrobrevin*$^{-/-}$ mice, AChR clusters are normal at birth but increasingly fragment postnatally. Similarly, in cultured *α-dystrobrevin*$^{-/-}$ myotubes, agrin induces normal AChR clustering, but these clusters are unstable and disperse rapidly when agrin is

withdrawn from the myotubes (Grady et al., 2000). Thus, the UGC is a core player in postnatal NMJ stabilization. Further candidates are Src-family kinases (SFKs). In *src*$^{-/-}$;*fyn*$^{-/-}$ mice, NMJs appear normal around birth, when the animals die. In cultured *src*$^{-/-}$;*fyn*$^{-/-}$ myotubes, agrin and laminin induce normal AChR aggregation, but the clusters disperse rapidly upon withdrawal of these factors (Smith et al., 2001; Marangi et al., 2002).

To elucidate the mechanisms of synaptic stabilization, we have investigated the role of SFKs in vivo. In adult myofibers expressing dominant-negative Src, AChR-rich areas were severely fragmented. We addressed the mode of SFK action in AChR cluster stabilization using *src*$^{-/-}$;*fyn*$^{-/-}$ myotubes. We found that Src and Fyn maintain clusters of rapsyn and UGC components, maintain AChR-rapsyn interactions and AChR β phosphorylation, mediate AChR-cytoskeletal linkage and control rapsyn protein levels. Our data introduce SFKs as critical players in NMJ stabilization in vivo and reveal that complex signaling pathways underlie stabilization.

Results

Role of SFKs: correctly balanced kinase activity is required to maintain adult NMJs in vivo

We addressed the role of SFKs in NMJ stabilization using an in vivo electroporation paradigm in soleus muscle of adult mice. This method allows one to import plasmids into synaptic and extrasynaptic regions of individual myofibers (Kong et al., 2004). Successfully electroporated fibers are identified by nuclear GFP-staining, since a GFP construct containing a nuclear localization signal is co-electroporated (Kong et al., 2004).

We first used a kinase-inactive Src expression construct, Src-AM, in this approach. Src-AM harbors two mutations, K295M and Y527F, which inactivate the kinase activity and the inhibitory C-terminal phosphorylation site, respectively. The resulting kinase-dead molecule preferentially adopts an open conformation and acts strongly in a dominant-negative way (Kaplan et al., 1994; Thomas and Brugge, 1997). Such dominant-negative Src constructs interfere with many members of the Src-family (e.g.

Src, Fyn and Yes), not just Src itself. The constructs therefore lead to reduction of cellular SFK function (Twamley-Stein et al., 1993; Roche et al., 1995).

Following electroporation of plasmids expressing siRNA against MuSK, postsynaptic AChR clusters are normal after two weeks but disassembled after 6 weeks (Kong et al., 2004). Apparently, adult NMJs are stabilized, presumably through multiple protein interactions and the cytoskeleton, such that it takes 6 weeks to see disassembly when a critical kinase - MuSK - is inactivated. Since SFKs are also tyrosine kinases, can interact with MuSK and act within the MuSK signaling pathway (Mohamed et al., 2001; Mittaud et al., 2004), we concentrated our analysis to 6 weeks after electroporation of Src-AM. Whole-mount preparations of myofibers were subjected to α-bungarotoxin (α-BT)-rhodamine, neurofilament and synaptophysin staining (in blue) and fluorescence microscopy (Fig. 1). In GFP-positive fibers (expressing Src-AM), AChR clusters often appeared partially disassembled: the typical "pretzel" shapes were disturbed in that they did not form one single continuous structure but contained substantial holes, fragmenting pretzels often into two or more subregions (Fig. 1A, middle). More dramatically, in some GFP-positive fibers NMJs were completely disassembled, so that no pretzels were observed at all but only small fragments of AChR-clusters (Fig. 1A, right). Confocal imaging confirmed these results and allowed, in 3-dimensional reconstruction, to better visualize the disassembly of AChR clusters in fibers expressing GFP and Src-AM (Fig. 1B). We again observed several degrees of fragmentation, ranging from partial (Fig. 1B, middle two columns) to complete disassembly (Fig. 1B, right). As controls, we used GFP-negative fibers in Src-AM experiments (Fig. 1A) or an empty expression vector lacking the Src-AM insert (Fig. 1B). In both cases, NMJs appeared mostly intact.

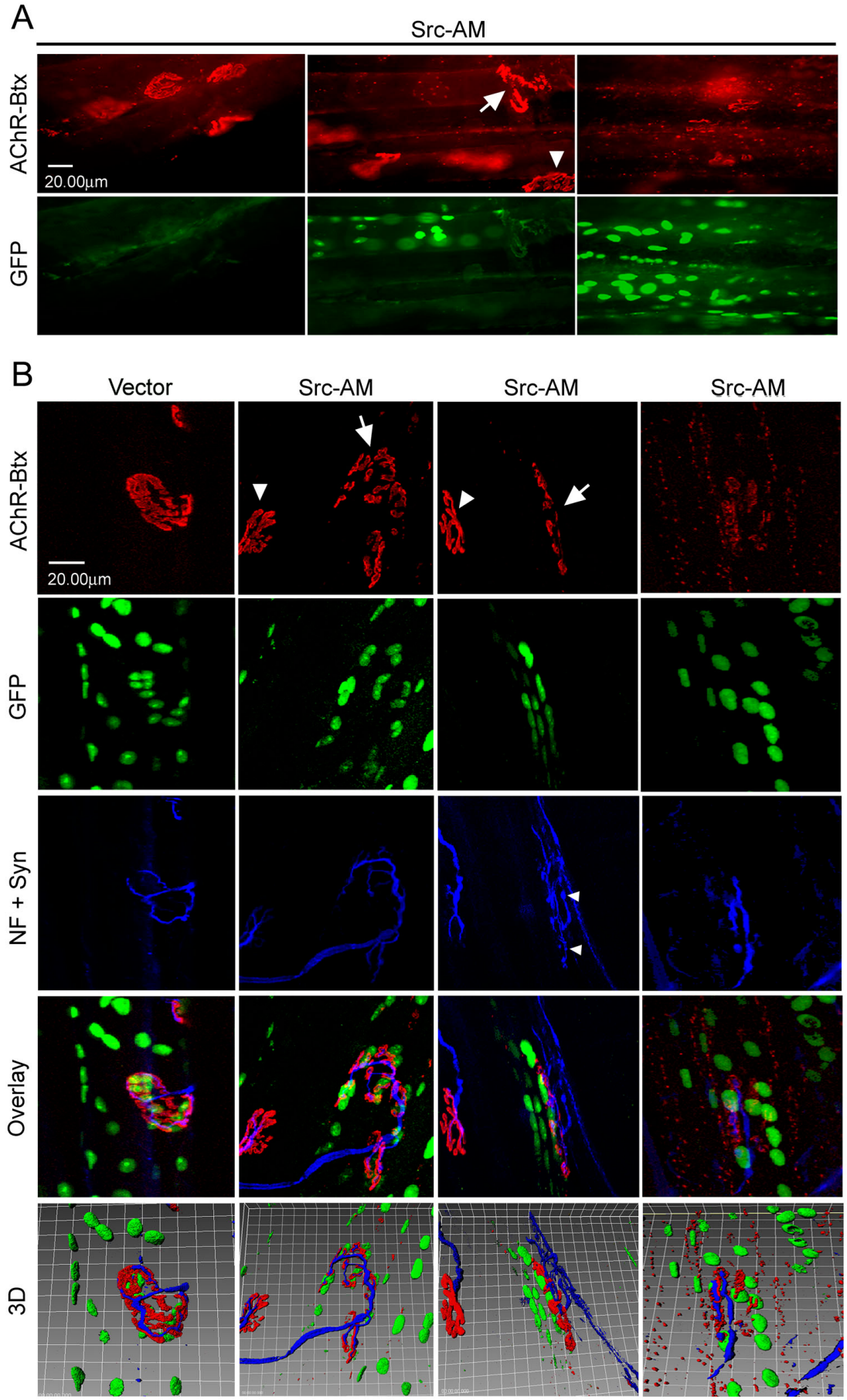

A
Src-AM
AChR-Btx
20.00µm
GFP
B
Vector
Src-AM
Src-AM
Src-AM
AChR-Btx
20.00µm
GFP
NF + Syn
Overlay
3D

Figure 1. Electroporation of kinase-inactive Src-AM into soleus muscle leads to disassembly of NMJs. Muscles were electroporated in vivo with a mixture of Src-AM and NLS-GFP or empty vector and NLS-GFP. 6 weeks later, muscles were dissected and whole mounts of fibers stained with α-BT-rhodamine and a mixture of neurofilament (NF) and synaptophysin (Syn) antibodies (in blue). (A) Conventional microscopy shows that in GFP-positive fibers NMJs are partially (middle, arrow) or completely (right) disassembled, while GFP-negative fibers show intact NMJs (left, and arrowhead in middle). (B) Confocal microscopy with 3D reconstruction to visualize the degree of NMJ disassembly and the topology of nerve, AChRs and synaptic myonuclei. Control vector electroporation leaves NMJs intact (left), while Src-AM electroporation disassembles NMJs in GFP-positive (arrows in middle columns) but not GFP-negative fibers (arrowheads in middle columns). Disassembly ranges from partial (middle columns) to complete (right). Occasionally, nerves of disassembled NMJs show sprouting as indicated by the small arrowheads. Clusters of synaptic nuclei are less dense in disassembled NMJs, and the topology of nerve versus AChRs is disturbed.

Rotation of 3D reconstructions using Imaris software revealed the typical architecture of intact NMJs in muscles electroporated with control empty vector: GFP-labeled synaptic nuclei were properly clustered underneath the AChR-pretzel, and AChRs mostly underneath the nerve (Fig. 1B and 2A-E). This topology was disturbed in fragmented NMJs in fibers expressing Src-AM. Here, remnants of AChR-pretzels were in the same focal plane as the nerve with no preferential labeling underneath it (confirmed by analysis of single confocal stacks; data not shown), such that the nerve was equally visible from a top view and a view from inside the muscle (Fig. 1B and 2F-J). In addition, clusters of synaptic nuclei became dispersed, following the fragmentation of the AChR-pretzels. In fragmented NMJs, we occasionally observed nerve labeling that resembled sprouting (Fig. 1B, middle Src-AM column). We quantitated the proportion of normal, partially and completely disassembled AChR clusters for Src-AM-expressing and control fibers. In the control, most endplates contained intact AChR-pretzels, some scored as partially disassembled clusters but we observed almost no completely disassembled cases (Fig. 2K). In Src-AM-expressing fibers, the proportion of partially and completely disassembled endplates was much higher (Fig. 2K).

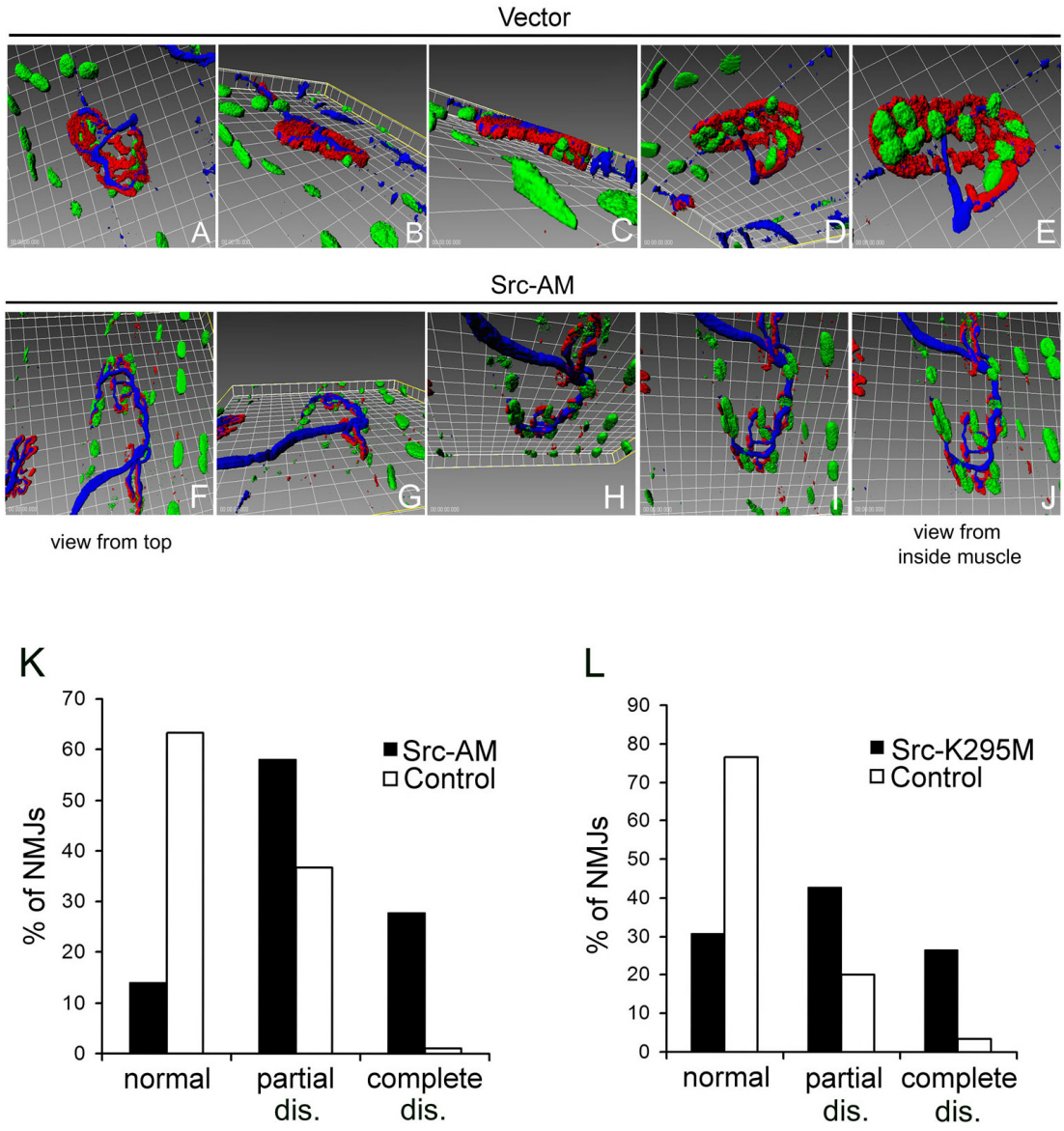


Figure 2. 3-dimensional rotation and quantitation of intact and disassembled endplates of muscle fibers expressing control plasmid or kinase-inactive Src. (A-J) 3D-reconstructions as shown in Fig. 1B were rotated around the x-axis to illustrate the relative positioning of nerve, AChR clusters and GFP-stained nuclei. (K and L) In experiments as described in Fig. 1, the degree of NMJ disassembly was scored as detailed in Materials and Methods. Control fibers score mostly as intact endplates with some partial and no complete disassembly. Fibers expressing Src-AM (K) or Src-K295M (L) show a high percentage of complete and partial disassembly as compared to control. Disassembly is quantified as percentage of all endplates analyzed within the control, Src-AM and Src-K295M groups. 50 endplates were analyzed for each group.

We repeated these experiments using another Src mutant, a construct that carried only a single mutation, K295M. This is the classic kinase-inactive, dominant-negative Src form, used most often to investigate the involvement of SFKs in signaling pathways (Kaplan et al., 1994; Roche et al., 1995; Thomas and Brugge, 1997). We observed results very similar to those with Src-AM (Fig. 2L). Taken together, these data demonstrate that normal SFK activity is a requirement to maintain the proper structure of adult NMJs in vivo.

In most cells, SFK activation is under tight regulation by a variety of extracellular signals and intracellular protein interactions (Thomas and Brugge, 1997). Experimentally, reducing or increasing SFK activity can produce changes in downstream signaling pathways (Thomas et al., 1995; Brandt et al., 2002; Kilarski et al., 2003). We therefore addressed whether increased SFK activity, leading to gain of SFK function, would disturb the postsynaptic organization of AChRs. For this purpose, we electroporated a Src construct, Src-Y527F, in which the inhibitory C-terminal phosphorylation site is replaced by phenylalanine. The resulting Src kinase is disinhibited and constitutively activated (Kaplan et al., 1994; Kilarski et al., 2003). In GFP-positive fibers, many AChR structures resembled perforated pretzels, containing holes or broken-up regions, very similar to partially disassembled endplates observed with Src-AM (Fig. 3A). Confocal 3-dimensional imaging illustrated the process of postsynaptic disassembly, revealing that synaptic nuclei were more dispersed, as the AChRs (Fig. 3B). Interestingly, in fibers expressing Src-Y527F we observed no completely disassembled AChR clusters, unlike for Src-AM. Quantitation showed that Src-Y527F expression strongly increased the proportion of partially disassembled AChR pretzels in comparison to control situations (Fig. 3C).

Taken together, the data demonstrate that proper regulation of kinase activity of SFKs in myofibers is a critical aspect in maintaining a normal morphology of the NMJ. If SFK activity is decreased, due to dominant-negative kinase-dead Src expression, postsynaptic organization is strongly affected, ranging from partial to complete disassembly of AChR-pretzels and alterations in the relative topology of nerve terminals, AChRs and synaptic nuclei. If SFK activity is increased, by expressing overactive Src, the morphology is also disrupted, albeit to a lesser degree.

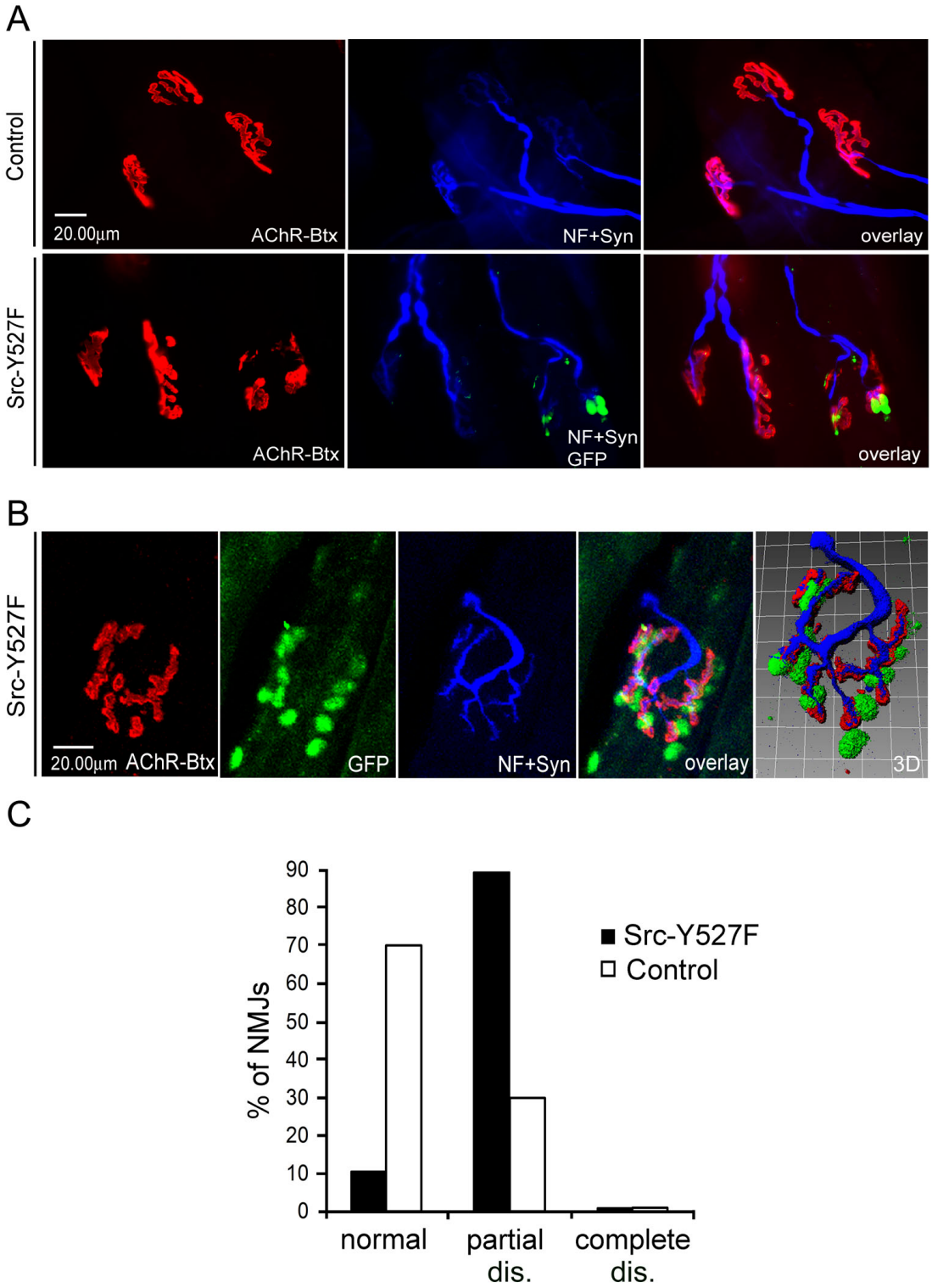


Figure 3. Electroporation of constitutively active Src-Y527F leads to partial disassembly of NMJs in vivo. Experiments were performed as for Figure 1 and 2. (A) Conventional microscopy shows that upon Src-Y527 electroporation, NMJs disassemble partially in GFP-positive fibers but not in GFP-negative fibers or in control muscles that were not electroporated. (B) Confocal analysis illustrates the

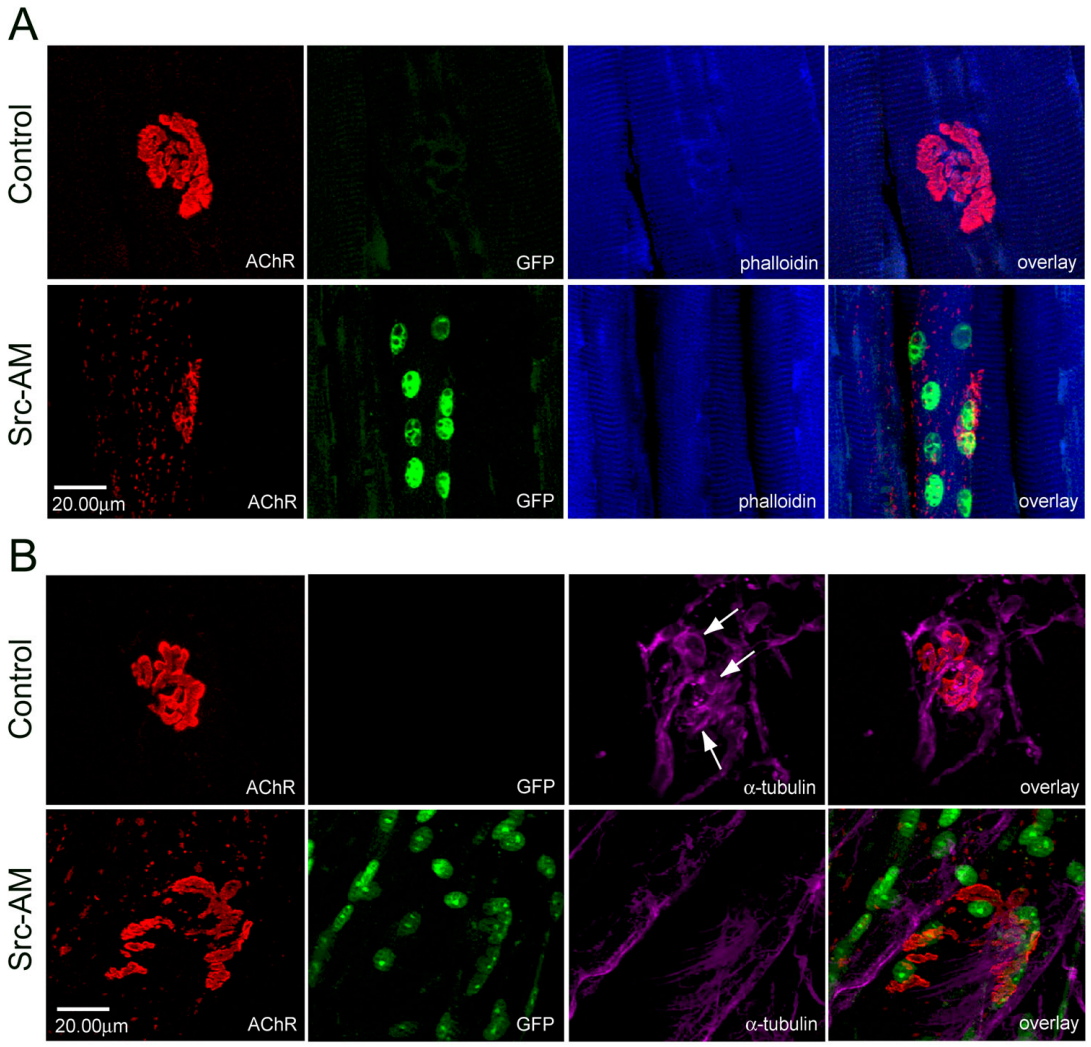
partial disassembly of an endplate, displaying large holes between AChR pretzel fragments. Nerves and synaptic nuclei are arranged accordingly. (C) Quantitation of 30 Src-Y527F and 30 control situations shows that Src-Y527F expression increases partial disassembly without leading to complete disassembly.

Expression of dominant-negative Src alters the subsynaptic but not extrasynaptic cytoskeleton and does not cause muscle degeneration

It was important to ascertain that Src-AM electroporation directly affects postsynaptic processes and not global muscle substrates that may indirectly lead to synaptic changes. We focussed on two cytoskeletal elements and known substrates of SFK-activated signaling cascades, F-actin and α-tubulin. In other cells, kinase-dead as well as kinase-overactive Src expression affects their organization (Cox and Maness, 1993; Thomas et al., 1995; Brandt et al., 2002; Kilarski et al., 2003). We stained whole-mount preparations with fluorescent phalloidin to visualize actin filaments and found a transverse pattern throughout myofibers. This is indicative of costameric structures (Rybakova et al., 2000), and there was no difference between Src-AM-expressing and control fibers that had disassembeld and intact NMJs, respectively (Fig 4A). α-tubulin stains revealed typical ring-like structures in the subsynaptic zone of the NMJ, as reported before (Ralston et al., 1999) (Fig. 4B, arrows). Analysis of single confocal sections located the rings subsynaptically underneath AChR clusters (data not shown). These rings often surrounded synaptic nuclei (Ralston et al., 1999) as verified in control experiments using empty vector and GFP (data not shown). Importantly, the rings were much less pronounced at disassembled endplates in fibers expressing Src-AM (Fig. 4B). The irregular extrasynaptic α-tubulin signal showed no difference between Src-AM-epressing and control fibers. These data show cytoskeletal changes at dispersed NMJs but not throughout the muscle fiber, strongly suggesting that Src-AM acts through a specific postsynaptic mechanism to disassemble NMJs.

It was also important to verify that Src-AM does not induce muscle degeneration followed by regeneration, a process that could indirectly contribute to postsynaptic disassembly at affected NMJs. A hallmark of muscle degeneration/regeneration is the appearance of myotubes with smaller diameter and centrally positioned nuclei (Paoni

et al., 2002). We analyzed cross-sections of electroporated muscle by triple-labeling with anti-GFP antibodies, rhodamie-α-BT and DAPI. This allowed to identify muscle fibers, due to diffuse GFP-signal, and the positioning of nuclei within those fibers, since most of the strong GFP signals overlapped with the nuclear DAPI signal (Fig. 4C, D). In myofibers expressing Src-AM or empty control vector, all GFP signals were always at the fiber periphery, never in the center (Fig. 4C). Also in fibers expressing Src-AM and having disassembled NMJs, nuclei were peripheral (Fig. 4D). Finally, we did not observe small diameter myotubes. Thus, electroporation with empty vector or Src-AM does not lead to detectable degeneration and regeneration of muscle, implying that Src-AM expression disrupts the postsynaptic NMJ apparatus by a more direct subsynaptic mechanism.



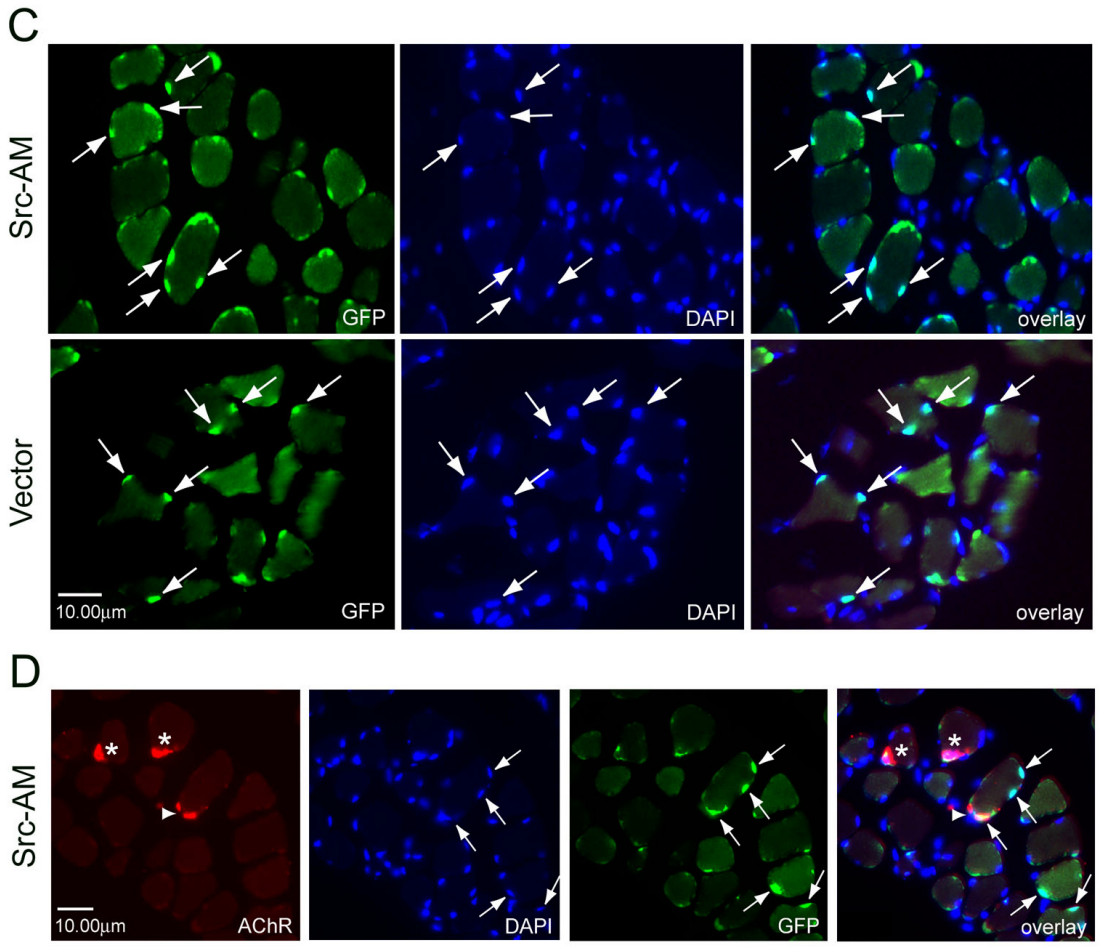


Figure 4. Electroporation of Src-AM causes specific cytoskeletal changes at the postsynapse and does not induce muscle degeneration/regeneration. (A, B) Muscles were electroporated with Src-AM and NLS-GFP, and whole mounts stained with rhodamine-α-BT and Alexa 350-coupled phalloidin (to visualize actin filaments)(A; blue) or antibodies against α-tubulin (B; pink). The organization of F-actin in costameric structures along myofibers is not affected in Src-AM-expressing, GFP-positive fibers in which NMJs are disassembled (A). Subsynaptic α-tubulin is organized in ring-like structures at intact NMJs (arrows in B), and this arrangement is disturbed at disassembled NMJs in GFP-positive fibers. (C) Muscles were electroporated with Src-AM and NLS-GFP, or empty control vector and NLS-GFP. Muscles were first processed and fixed as for whole-mount analysis, but then embedded, cryosectioned, and stained with anti-GFP antibodies and DAPI as described in Materials and Methods. Muscles fibers are visible due to low-intensity diffuse GFP-staining. In both Src-AM and vector samples, strong GFP signals are always at the fiber periphery and mostly colocalize with DAPI, identifying them as peripheral nuclei (arrows). Thus Src-AM does not lead to centrally positioned nuclei, excluding the presence of myotubes and degeneration/regeneration. (D) Samples as in C were additionally stained with rodamine-α-BT. Fibers lacking nuclear GFP-signal show intact AChR

clusters (asterisks), while a fiber with disassembled AChR clusters (arrowhead) displays peripheral nuclei (arrows).

Mechanism of SFK action: they do not act in recruitment but in stabilization of postsynaptic proteins in clusters

We examined the postsynaptic mode of action of SFKs in stabilizing AChR clusters, by using cultured *src*$^{-/-}$;*fyn*$^{-/-}$ myotubes for detailed cell biological and biochemical analysis. These cells are a useful model for postsynaptic stabilization: synapse-promoting factors such as neural agrin or laminin induce normal AChR clustering, but most clusters disassemble when these factors are withdrawn from the cell medium for a few hours, while clusters in wild-type cells remain stable (Smith et al., 2001; Marangi et al., 2002).

We first addressed whether SFKs act in stabilization of agrin-induced AChR clusters by recruiting other proteins into AChR-containing aggregates. We analyzed many UGC components, since this complex plays a central role in postsynaptic stabilization and maturation (Grady et al., 1997; Grady et al., 2000; Jacobson et al., 2001). We treated cultured *src*$^{-/-}$;*fyn*$^{-/-}$ myotubes with agrin and examined them by immunocytochemical staining and fluorescence microscopy. Utrophin, α-dystrobrevin, α-dystroglycan, syntrophin, rapsyn and phosphotyrosine-containing proteins were all clustered normally by agrin treatment, and all proteins efficiently colocalized with AChRs (Fig. 5). Recruitment of these proteins into AChR-containing clusters occurs thus independently of Src and Fyn.

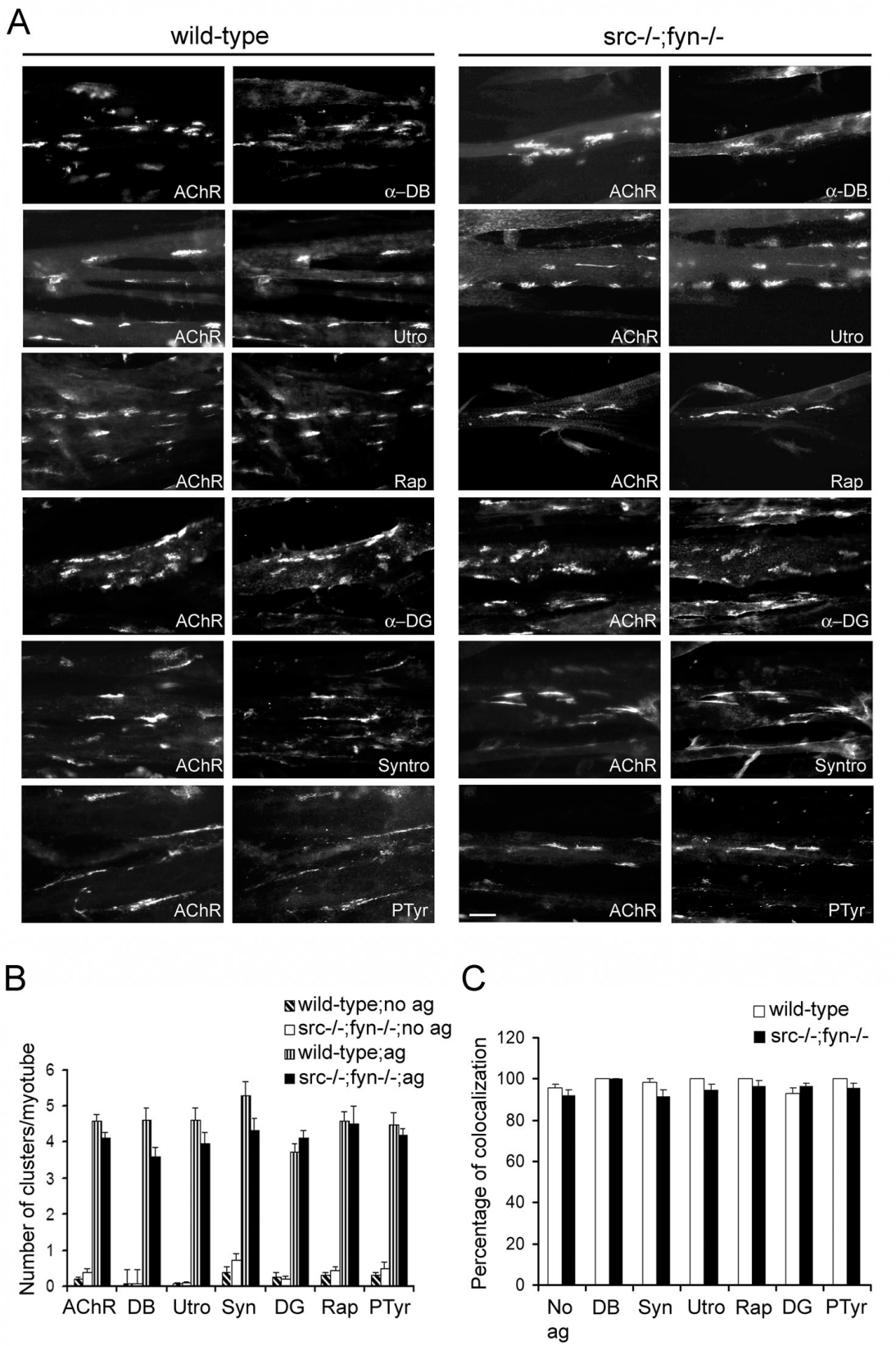
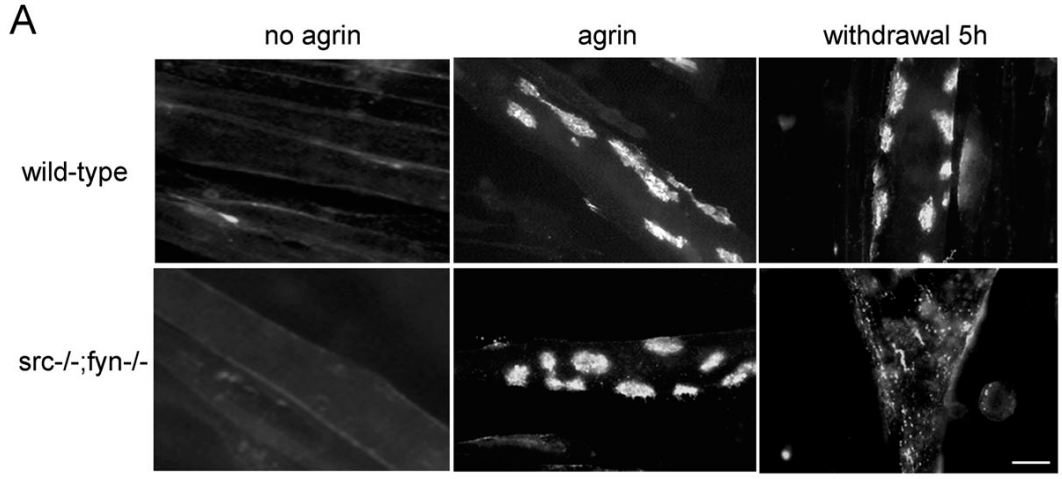

A
wild-type
src-/-;fyn-/-
AChR
α–DB
Utro
Rap
α–DG
Syntro
PTyr
B
wild-type;no ag
src-/-;fyn-/-;no ag
wild-type;ag
src-/-;fyn-/-;ag
Number of clusters/myotube
AChR DB Utro Syn DG Rap PTyr
C
wild-type
src-/-;fyn-/-
Percentage of colocalization
No ag DB Syn Utro Rap DG PTyr

Figure 5. Agrin induces co-clustering of postsynaptic proteins with AChRs in src$^{-/-}$;fyn$^{-/-}$ myotubes. (A) Cells were incubated with 0.5 nM agrin overnight and double-labelled with rhodamine-α-BT (AChR) and antibodies recognizing α-dystrobrevin-1 (α-DB), utrophin (Utro), rapsyn (Ross et al.), α-dystroglycan (α-DG), syntrophin isoforms (Syntro) or phosphotyrosine (PTyr), followed by FITC-coupled secondary antibodies. Wild-type and src$^{-/-}$;fyn$^{-/-}$ myotubes show identical co-clustering of AChRs with the respective postsynaptic proteins. Scale bar = 10 μm. (B) Clusters of AChRs and postsynaptic proteins were counted in agrin-treated (ag) and untreated (no ag) cells. (C) Protein colocalization is expressed as % of AChR clusters that contain the respective postsynaptic protein in agrin-treated cells. "No ag"shows the average of colocalization of each marker with AChRs in non agrin-treated cells. Values are mean ± SEM, from 20 pictures for each marker and condition.

We next asked whether SFKs act in stabilizing clusters of postsynaptic proteins, by treating *src$^{-/-}$;fyn$^{-/-}$* myotubes with agrin to induce aggregates. Agrin was then withdrawn for 4-5 h to assess the stability of these clusters. Utrophin clusters disappeared rapidly in parallel with AChR aggregates: the number of remaining utrophin clusters was as low as the number of AChR aggregates after 4 h withdrawal, and remaining AChR clusters colocalized efficiently with utrophin (Fig. 6A, B and Figure 7. 1). We observed the same for α-dystrobrevin (Fig. 6C) and rapsyn (Fig. 6D)(see also Figure 7. 1). These data demonstrate that SFKs are not essential in the formation of clusters of postsynaptic proteins. Rather SFKs are required for stabilization of AChRs and all other proteins examined in co-extensive clusters, illustrating a general importance of SFKs in postsynaptic maintenance by holding together the postsynaptic apparatus.



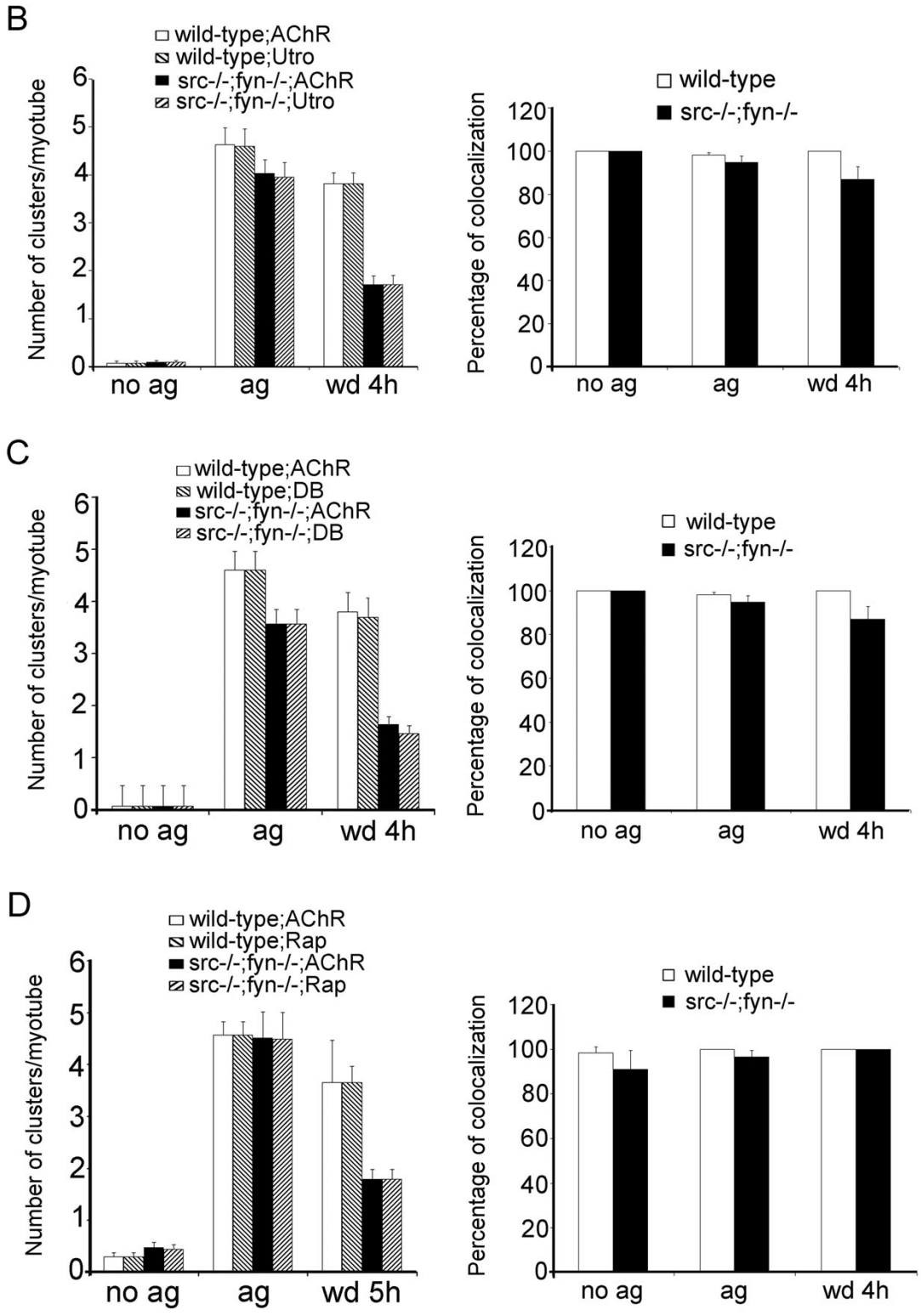


Figure 6. Src and Fyn are required to stabilize postsynaptic protein clusters along with AChR clusters. Cells were treated overnight with 0.5 nM agrin to induce protein clustering. For studying cluster maintenance, agrin was then withdrawn from cultures for 4-5 hrs (wd 4h, wd 5h). (A) Cells were

labelled with rhodamine-α-BT (AChR), showing that 5 hrs of withdrawal causes pronounced AChR cluster disassembly in src-/-;fyn-/- but not wild-type myotubes. Cells were double-labelled with rhodamine-α-BT (AChR) and antibodies recognizing utrophin (B), α-dystrobrevin-1 (C), or rapsyn (D), followed by FITC-coupled secondary antibodies. Illustrative pictures of clusters and their disassembly are shown in Figure 7. 1. Quantitation (left) shows that clusters of these postsynaptic proteins disappear in parallel with AChRs upon agrin withdrawal. Colocalization (right) indicates % of AChR clusters that contain the respective postsynaptic protein, in the case of agrin withdrawal for remaining AChR clusters. Values are mean ± SEM, from 20 pictures for each marker and condition.

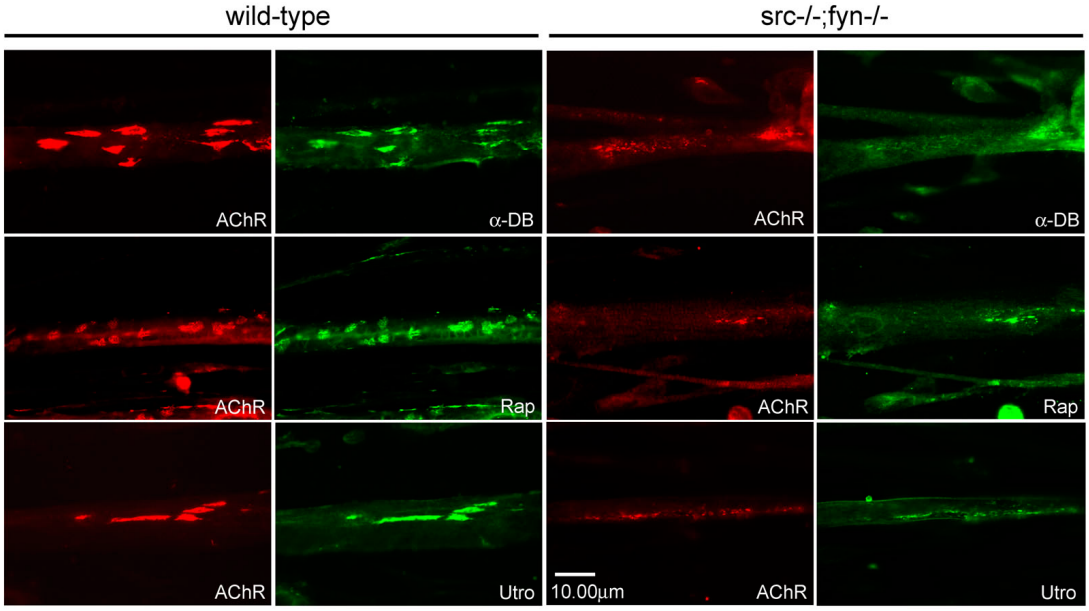


Figure 7. Myotubes were incubated overnight with 0.5 nM agrin following which agrin was withdrawn for 5 h. Cells were double labeled for the AChR with α-BT-rhodamine and for the respective postsynaptic proteins. In $src^{-/-};fyn^{-/-}$ myotubes clusters of α-dystrobrevin-1 (α-DB), rapsyn (Ross et al.), and utrophin (Utro) disperse in parallel with the AChR clusters. In wild-type cells clusters of AChRs and postsynaptic proteins remain stable. Scale bar, 10 µm.

SFKs maintain agrin-induced AChR-rapsyn interaction

To unravel the mechanisms by which SFKs maintain clusters of AChRs and other postsynaptic proteins, we analyzed AChR-protein interactions biochemically in *$src^{-/-};fyn^{-/-}$* myotubes, focusing first on rapsyn. Rapsyn is a key anchor for the AChR in clusters (Gautam et al., 1995) and its interaction with the receptor is highly

regulated and increased by agrin (Moransard et al., 2003). Rapsyn exists in free and AChR-bound form (Marangi et al., 2001; Moransard et al., 2003). While intracellular and unclustered surface AChRs interact with some rapsyn, agrin induces increased rapsyn-AChR binding at the plasma membrane, and this increase tightly correlates with clustering and cytoskeletal anchoring. Likewise, synaptic AChRs contain more bound rapsyn than extrasynaptic AChRs in denervated muscle (Moransard et al., 2003). Since increased rapsyn-AChR interaction thus underlies clustering, we analyzed this interaction in cluster formation and stabilization in the absence of Src and Fyn. We treated *src*$^{-/-}$;*fyn*$^{-/-}$ myotubes with agrin and then withdrew agrin for 3 or 5 hrs (Fig. 8A). AChRs were precipitated from lysates using biotinylated α-BT and streptavidin-agarose (Tox-P), and AChR-bound rapsyn was visualized by immunoblotting. Interestingly, in untreated *src*$^{-/-}$;*fyn*$^{-/-}$ myotubes, α-BT-precipitated AChRs contained in average more associated rapsyn than in wild-type cells (Fig. 8A, B). This interaction was further increased ca. two-fold by agrin treatment, but returned to basal levels within 5 h of agrin withdrawal. In contrast, rapsyn binding to AChR in wild-type cells, increased by agrin to the same relative degree (ca. two-fold) as in the mutant, remained increased even after 5 h withdrawal (Fig. 8A, B). Thus, although AChRs contain in average more associated rapsyn in *src*$^{-/-}$;*fyn*$^{-/-}$ myotubes, the agrin-induced increase in the interaction is very unstable in the absence of Src and Fyn.

We previously quantitated the degree of rapsyn co-precipitation with AChRs and found that not every precipitated receptor molecule is associated with one rapsyn molecule (Fuhrer et al., 1999). Other observations suggested that rapsyn exists in an equilibrium between free and AChR-associated state (Marangi et al., 2001; Moransard et al., 2003) and that the average ratio of rapsyn to AChR expression is ca. 1:1 (LaRochelle and Froehner, 1987). The extraction step of our α-BT precipitation of AChRs is likely to break up some AChR-rapsyn-interaction (Fuhrer et al., 1999). For these reasons, the higher basal degree of rapsyn co-precipitation with AChRs in *src*$^{-/-}$;*fyn*$^{-/-}$ myotubes may originate from a weakened cytoskeletal linkage of rapsyn, making its extraction and co-precipitation with the receptor more efficient. Alternatively, rapsyn protein may be present in higher amount in the mutant cells, so

that more rapsyn is available to bind the receptor in the absence of Src and Fyn. To distinguish between these two possibilities, we analyzed two aspects of rapsyn in *src*$^{-/-}$;*fyn*$^{-/-}$ myotubes.

First, we determined the amount of rapsyn protein in cellular lysates. Using immunoblotting, we observed higher amounts of rapsyn protein per milligram of cellular protein in standard lysates, made with 1% detergent, of *src*$^{-/-}$;*fyn*$^{-/-}$ cells as compared to wild-type cells (Fig. 8C and D). In many further extractions using milder and harsher conditions (decreasing or increasing detergent and salt concentrations), we always found similarly elevated rapsyn protein levels in *src*$^{-/-}$;*fyn*$^{-/-}$ cells (data not shown). As control, we quantitated the cellular amounts of other postsynaptic proteins, such as utrophin, MuSK and α-dystrobrevin. We observed no significant difference in these proteins between mutant and wild-type cells (data not shown), suggesting that the effect of Src and Fyn on rapsyn levels is specific.

Second, we measured the relative strength of cytoskeletal rapsyn interaction. We performed extraction experiments similar to those described in detail for AChRs in Figure 10, employing low and high detergent concentrations. By immunoblotting we determined the proportion of rapsyn in each of these extractions. The proportion of rapsyn in low versus high detergent extractions was the same for *src*$^{-/-}$;*fyn*$^{-/-}$ and wild-type myotubes (data not shown), showing that the cytoskeletal link of rapsyn is unchanged in the mutant.

Taken together, these data, combined with earlier observations (Marangi et al., 2001; Moransard et al., 2003), strongly imply that since *src*$^{-/-}$;*fyn*$^{-/-}$ myotubes contain elevated levels of rapsyn protein, rapsyn associates to a higher overall degree with the AChR. But the agrin-induced rapsyn interaction is very unstable following agrin withdrawal from mutant cells. This instability parallels the concomitant disappearance of AChR and rapsyn clusters (Fig. 6). Thus stabilization of rapsyn-AChR interaction appears as primary mechanism by which SFKs hold together the postsynaptic apparatus.

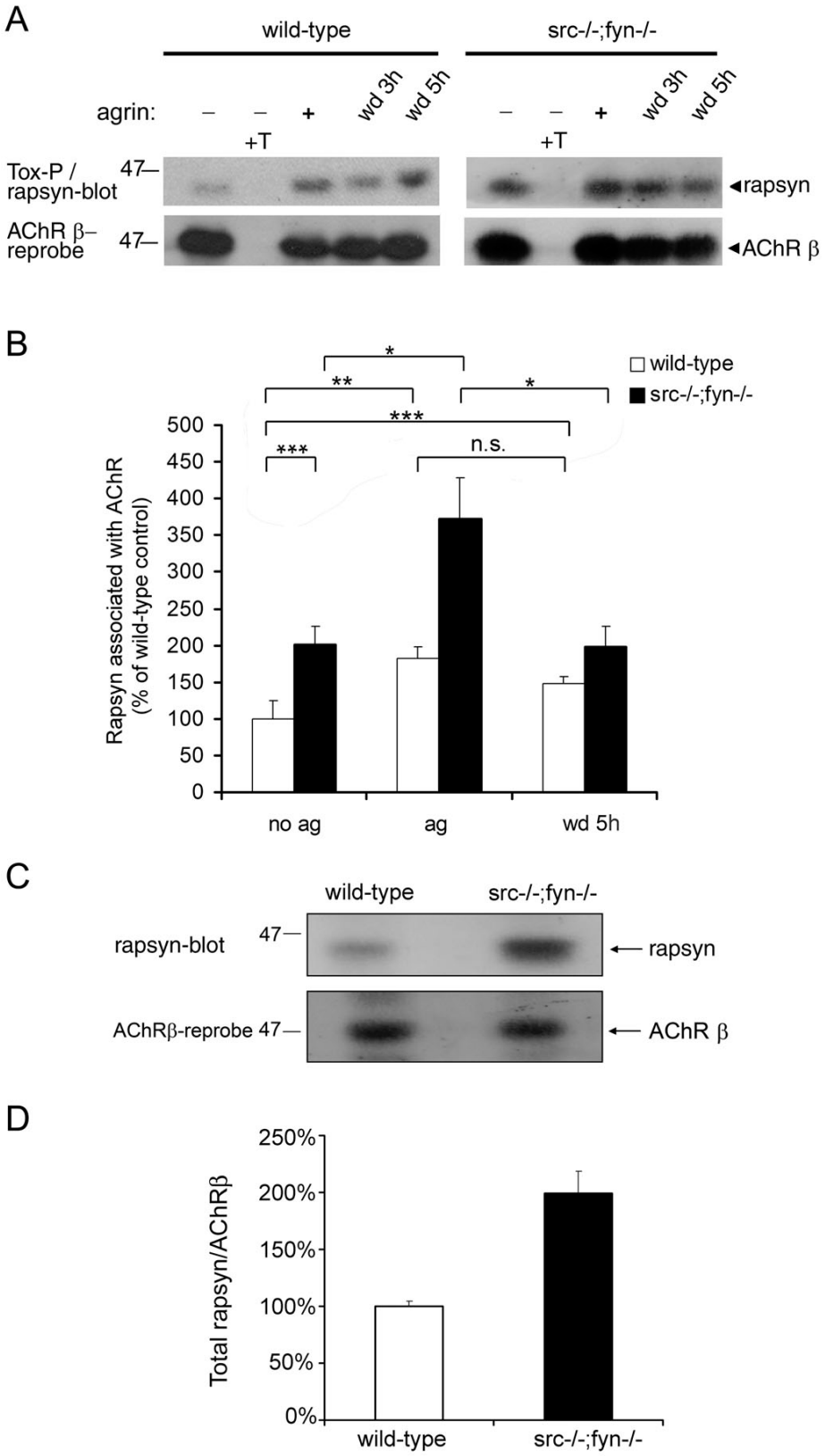


Figure 8. SFKs maintain AChR-rapsyn interaction and negatively regulate rapsyn protein levels. (A) Cells were treated overnight with 0.5 nM agrin followed by withdrawal (wd) as indicated. AChRs were

precipitated from lysates using α-BT-biotin and streptavidin-agarose (Tox-P), and associated rapsyn was detected by Western blotting. As control, an excess (10 μM) of free soluble toxin (+T) was added to some lysates. In the bottom part, the blots were stripped and reprobed for the AChR β subunits with mAb 124. (B) Experiments were quantitated by densitometric scanning. Rapsyn signals were divided through AChR β signals to ensure equal loading. The graph indicates the % of rapsyn associated with AChR, with wild-type cells not treated with agrin set to 100%. Untreated *src*$^{-/-}$;*fyn*$^{-/-}$ myotubes show an increased rapsyn association (ca. 2-fold) with the AChR. Agrin further increases this interaction about 2-fold, similarly to wild-type cells. In *src*$^{-/-}$;*fyn*$^{-/-}$ myotubes the agrin-induced increase in rapsyn-AChR interaction decreases after agrin withdrawal but remains more stable in the wild-type. Data represent mean ± SEM of at least 5 experiments. *p<0.05, **p<0.01, ***p<0.001, by unpaired Student's t-tests (n.s., not significant). (C) Protein-matched aliquots of wild-type and *src*$^{-/-}$;*fyn*$^{-/-}$ myotube lysates were analyzed by immunoblotting using antibodies against rapsyn (Rap-1) and AChR β subunits (mAb 124). (D) Quantitation shows that the level of rapsyn protein (determined as rapsyn/AChRβ) is two-fold higher in mutant cells.

SFKs maintain AChR-dystrobrevin interaction and AChR phosphorylation

In the process of clustering, agrin increases some but not all interactions of AChRs with other proteins (Fuhrer et al., 1999). We investigated whether α-dystrobrevin, a UGC component essential for stabilization of the postsynaptic membrane and AChR aggregates (Grady et al., 2000), increasingly associates with AChRs following agrin treatment and withdrawal. We focused on the α-dystrobrevin-2 isoform, since our available antibodies best allowed detection of this form. Precipitation with α-BT and α-dystrobrevin-2 immunoblotting revealed that agrin induces increased binding of α-dystrobrevin-2 to AChRs in both wild-type and *src*$^{-/-}$;*fyn*$^{-/-}$ myotubes (Fig. 9A). Following withdrawal of agrin, however, less α-dystrobrevin-2 remained bound to the receptor in the mutant as compared to wild-type cells (Fig. 9A), showing that Src and Fyn are required for optimal stabilization of AChR-UGC interaction. This parallels the stabilization by SFKs of AChR-rapsyn interaction, consistent with the idea that rapsyn is a linker between AChR and the UGC (Apel et al., 1995; Cartaud et al., 1998; Bartoli et al., 2001).

Phosphorylation of the AChR β subunit is a key event in agrin signaling and important for efficient clustering and cytoskeletal linkage of the receptor (Borges and

Ferns, 2001). Agrin-induced AChR β phosphorylation is an early step in agrin signaling and precedes receptor clustering, similar to agrin-triggered rapsyn-AChR interaction (Ferns et al., 1996; Moransard et al., 2003). We examined whether SFKs are required to maintain this phosphorylation in the process of cluster stabilization. Precipitation with biotin-α-BT and immunoblotting with anti-phosphoytrosine showed that agrin treatment caused normal β phosphorylation in *src*$^{-/-}$;*fyn*$^{-/-}$ myotubes, as described previously (Smith et al., 2001). But following agrin withdrawal, this phosphorylation was unstable and disappeared much faster than in wild-type cells (Fig. 9B). While in the wild-type, significant β phosphorylation persisted 5 h after agrin withdrawal, β phosphorylation was already down at basal levels after 3 h in *src*$^{-/-}$;*fyn*$^{-/-}$ myotubes (Fig. 9B). We thus estimate the half-life time ($t_{1/2}$) of β phosphorylation to be about 4 h in the wild-type but less than 90 min in the mutant. Since AChRs can be direct substrates for SFKs (Swope and Huganir, 1993; Fuhrer and Hall, 1996; Mohamed et al., 2001; Mittaud et al., 2004), these data strongly imply that SFK activity is required to maintain phosphorylation of the AChR, and that the receptor is a direct substrate for SFKs at that stage.

The correlation between loss of AChR phosphorylation and AChR-rapsyn binding following agrin removal raises the possibility that β phosphorylation may regulate rapsyn interaction. Collectively, the sum of our data on *src*$^{-/-}$;*fyn*$^{-/-}$ myotubes up to this point suggests that SFK-mediated stabilization of AChR phosphorylation and of AChR association with rapsyn and dystrobrevin form a core mechanism by which SFKs hold together proteins of the postsynaptic apparatus.

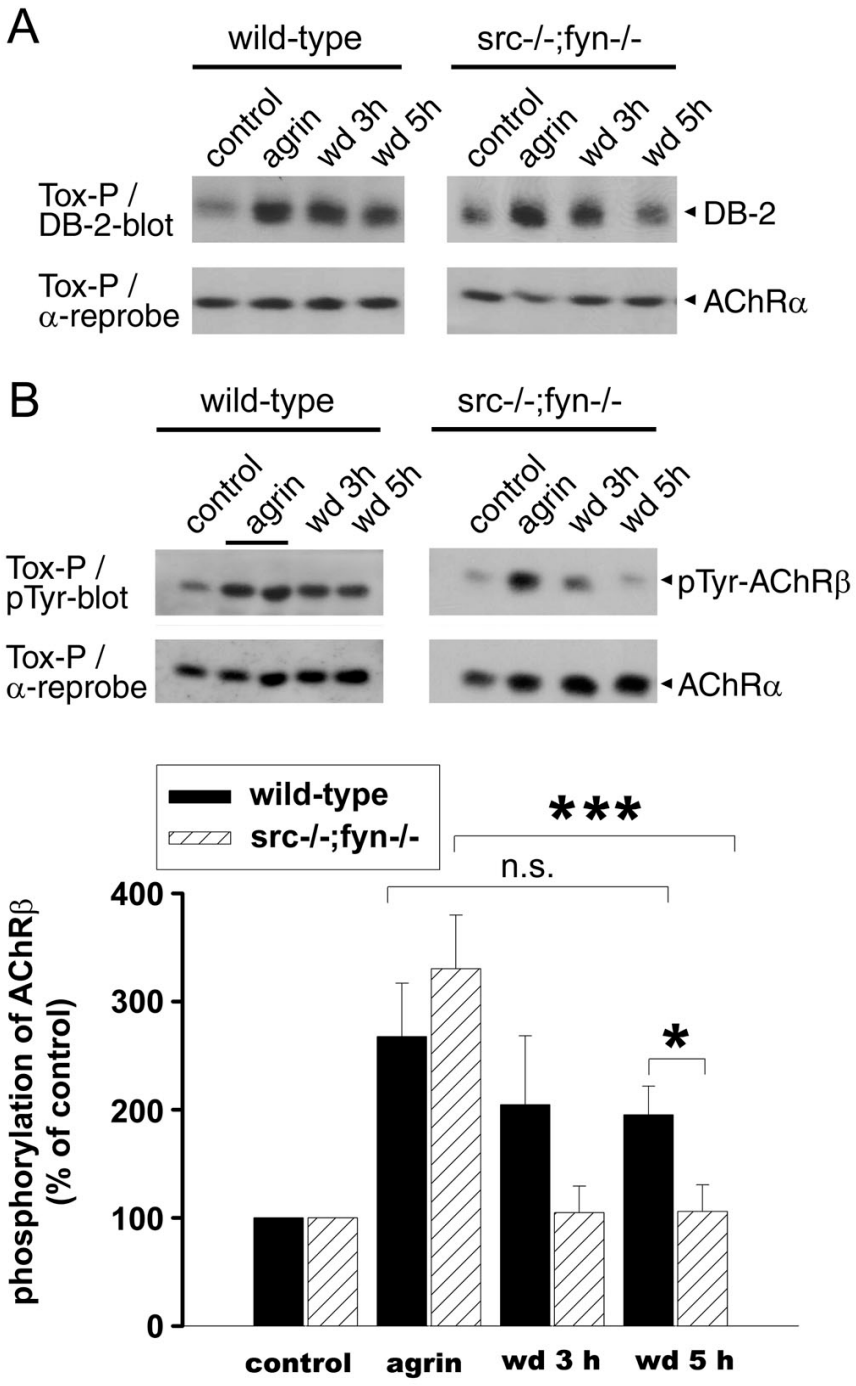


Figure 9. In *src*$^{-/-}$*;fyn*$^{-/-}$ myotubes, AChR-dystrobrevin association and phosphorylation of AChR β subunits are unstable after agrin withdrawal. Agrin was added overnight, cells were washed, and incubated in withdrawal medium (wd) lacking agrin for 3 or 5 hours. AChRs were precipitated with α-BT and subjected to immunoblotting using antibodies against α-dystrobrevin-2 (A) or against phosphotyrosine (B). Blots were stripped and reprobed for AChR α subunits as control. (A) Association of AChRs with α-dystrobrevin is increased by agrin in both wild-type and mutant cells.

After 5 hrs agrin withdrawal, the association is much weaker in the mutant than in the wild-type. (B) In mutant cells, phosphorylation of AChR β subunits - detected based on molecular weight and comparison to parallel AChR β immunoblots (not shown) - is normally induced by agrin but rapidly decreases after withdrawal. Quantitation of the phospho-AChR β signal by densitometric scanning, normalized for AChR α, shows a significant decrease in the mutant but not in the wild-type upon agrin withdrawal (mean ± SEM from at least 5 experiments; ***p=0.003; *p=0.018; n.s.: not significant; by unpaired Student's t-tests).

The overall link of AChRs to the cytoskeleton is weak in src$^{-/-}$;fyn$^{-/-}$ myotubes but still strengthened by agrin

In addition to changes at the level of AChR clusters, the alterations in postsynaptic architecture in Src-AM-expressing myofibers in vivo suggest that the postsynaptic cytoskeleton (as shown for α-tubulin in Fig. 4B) and its protein interactions may be altered upon loss of SFK function. To further test this hypothesis, we analyzed the role of SFKs in interactions of the AChR with the cytoskeleton. We quantitated the AChR extractability in *src*$^{-/-}$*;fyn*$^{-/-}$ myotubes, by applying a sequential detergent extractability protocol in which a first extraction in low detergent is followed by a second extraction in higher detergent concentration. From each extraction, we precipitated AChRs using biotin-α-BT, visualized them in Western blots, and calculated the receptor distribution between first and second extraction, using increasing detergent concentrations in the first extraction (Fig. 10A, B). Such methods are established measures for the relative strength of the interaction of the AChR to the cytoskeleton (Borges and Ferns, 2001; Moransard et al., 2003). We found that in wild-type myotubes, AChR started to become efficiently solubilized in the first extraction when the concentration of first detergent was 0.06% Triton X-100 (Fig. 9A). At lower Triton X-100 concentrations in the first extraction, receptors appeared in the second extraction, while at higher concentrations, AChRs participated into the first extraction (Fig. 9A). In contrast, in *src*$^{-/-}$*;fyn*$^{-/-}$ myotubes, a concentration of 0.04% Triton X-100 was sufficient to efficiently solubilize AChRs in the first extraction (Fig. 10B). These data reveal that the overall basal cytoskeletal link of the AChR is weakened in the absence of Src and Fyn.

Agrin treatment is known to strengthen the AChR-cytoskeletal link in the process of cluster formation (Wallace, 1992, 1995; Borges and Ferns, 2001; Moransard et al., 2003). This is manifested as a decrease of solubilized and precipitated AChR in the first extraction and an increase of receptor in the second extraction (Borges and Ferns, 2001; Moransard et al., 2003). Likewise, AChRs increasingly become cytoskeletally anchored at developing NMJs in vivo (Dennis, 1981; Slater, 1982). We analyzed whether agrin affects the weak overall cytoskeletal interaction of the AChR in *src*$^{-/-}$*;fyn*$^{-/-}$ myotubes, by quantitating the relative amounts of AChRs in the first and second extraction in agrin-treated and untreated cells. Agrin caused a shift of receptors from the first (0.05% Triton X-100) to the second (1% Triton X-100) extraction in *src*$^{-/-}$*;fyn*$^{-/-}$ myotubes (Fig. 10C and D), similar to results previously seen in wild-type cells (Borges and Ferns, 2001; Moransard et al., 2003). The percentage of agrin-induced decrease of AChR precipitated from the first extraction was identical between C2 myotubes, wild-type myotubes and *src*$^{-/-}$*;fyn*$^{-/-}$ myotubes (Fig. 9E).

Together, these results show that while the overall basal cytoskeletal linkage of AChRs is weaker in the absence of Src and Fyn, agrin treatment still strengthens this link. This is consistent with the normal phosphorylation and cluster formation of AChRs in agrin-treated src$^{-/-}$;fyn$^{-/-}$ myotubes and the decreased stability of such clusters following removal of agrin (Smith et al., 2001). Most likely, the agrin-induced strengthening of the AChR cytoskeletal link in mutant cells originates from linkage of the receptor to the UGC, since AChR-dystrobrevin-interactions are normally induced by agrin in these cells (Fig. 9A), and since the UGC interacts with F-actin (Winder et al., 1995).

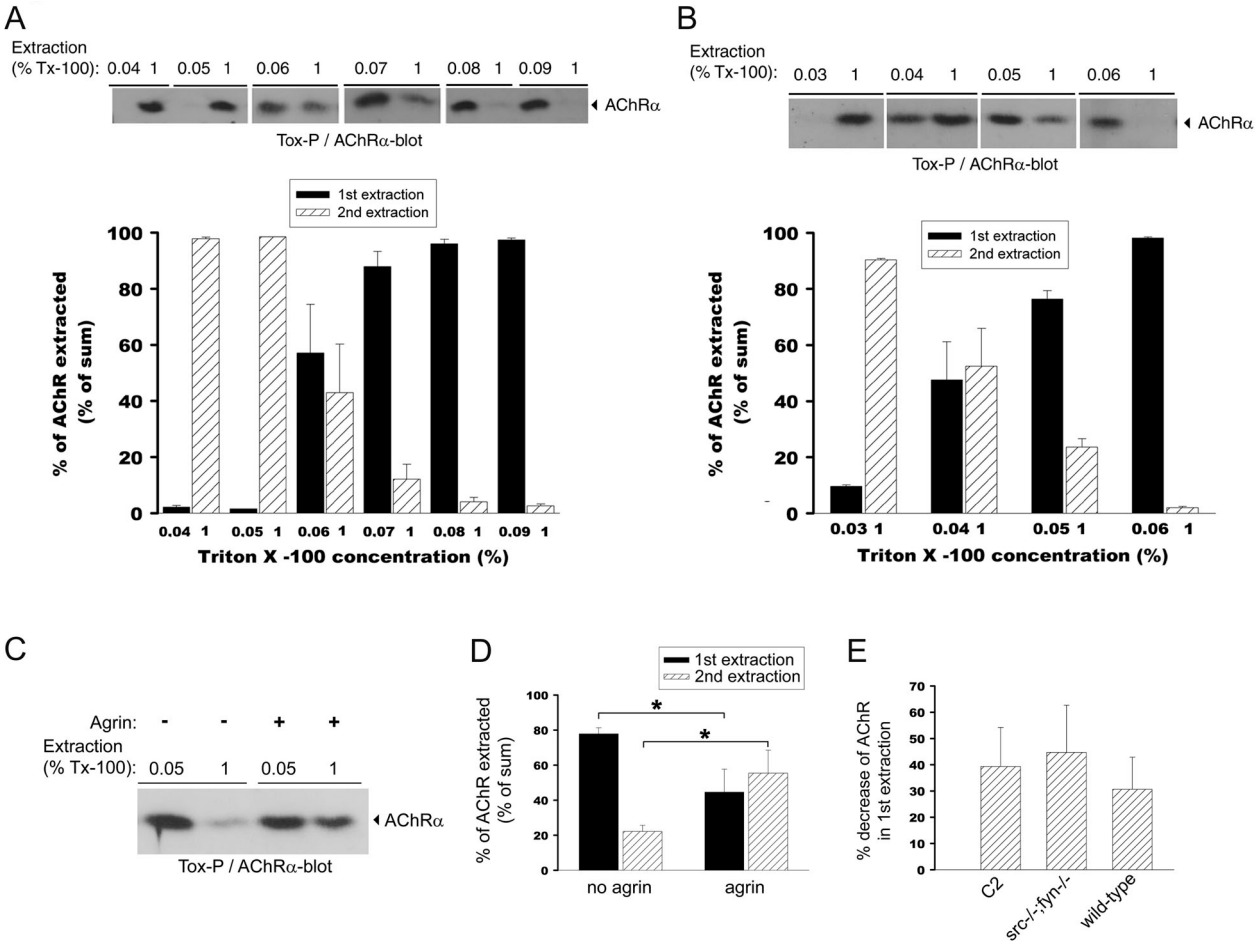


Figure 10. The overall basal cytoskeletal link of AChRs is weakened in *src*$^{-/-}$;*fyn*$^{-/-}$ myotubes but still strengthened by agrin treatment. Wild-type (A) or *src*$^{-/-}$;*fyn*$^{-/-}$ myotubes (B) were subjected to a first extraction in a buffer containing a low detergent concentration, ranging from 0.03 to 0.09% Triton X-100 as indicated. Insoluble materials (pellets) were subjected to a second extraction, using 1% Triton X-100. AChRs were precipitated from the soluble low- and high-detergent fractions using α-BT and visualized by anti-AChR α subunit-antibodies in immunoblots. AChR extraction was quantified by densitometric scanning, and AChR signals are shown as % of the sum of both extractions (low + high detergent). Values are mean ± SEM from at least 5 experiments. For wild-type cells, 0.06% detergent extracts about half of the total AChRs, while for *src*$^{-/-}$;*fyn*$^{-/-}$ myotubes only 0.04% detergent are required to achieve the same, indicating that in the mutant the AChR extractability is higher and thus the cytoskeletal link weaker. (C) Mutant cells were incubated overnight with agrin to induce AChR cluster formation. AChRs were first extracted with 0.05% and then with 1% detergent as described above, and visualized by α-BT precipitation and AChR α immunoblotting. Agrin causes decreased AChR extractability, indicating stronger cytoskeletal linkage, because less AChRs are found in the first extraction and more in the second. (D) Quantitation of experiments as in (C) shows a significant agrin-induced decrease of AChRs in the first extraction and a significant increase in the second extraction.

Values are mean ± SEM from 5 experiments, *p=0.03, by unpaired Student's t-tests. (E) Sequential extraction as in (C) was performed for wild-type and C2C12 myotubes, using 0.06% Triton X-100 in the first extraction and 1% in the second. The % of decrease of AChRs in the first extraction, induced by agrin, was quantitated from 5 experiments and is the same, ca. 30-40%, as for *src*$^{-/-}$;*fyn*$^{-/-}$ myotubes. Thus while the overall cytoskeletal link is weaker in *src*$^{-/-}$;*fyn*$^{-/-}$ myotubes, the agrin-induced strengthening of this link is comparable to wild-type cells.

Discussion

Our data reveal that SFKs are key players in the pathways that stabilize the postsynaptic apparatus of the NMJ in vivo. They act by holding postsynaptic proteins together in clusters through stabilization of rapsyn-AChR interaction and AChR phosphorylation. In addition, they control rapsyn protein levels and AChR-cytoskeletal linkage.

SFKs hold together the postsynaptic apparatus

Interference with SFK function causes complex alterations at adult NMJs. Upon Src-AM expression, AChR pretzels fragment and attach to the nerve in the same focal plane (not underneath it), subsynaptic α-tubulin organization is disturbed, synaptic nuclei become more dispersed and nerves occasionally sprout. These changes originate from Src-AM expression in muscle, acting specifically on postsynaptic mechanisms: GFP-positive nuclei were only seen in myofibers and never in other cells (e.g. Schwann cells), confirmed by 3D-reconstruction (G. Sadasivam and C. Fuhrer, unpublished observations), and costameric F-actin organization along myofibers was not affected. Nerve sprouting is in accordance with studies showing that postsynaptic disturbance affects the nerve, leading to sprouting or, as is the case in rapsyn- or MuSK-deficient mice, extensive nerve growth (Gautam et al., 1995; DeChiara et al., 1996; Kong et al., 2004).

The effects of Src-Y527F expression are similar, illustrating that correctly balanced SFK activity is important to maintain the postsynaptic apparatus in vivo. Consistent with this, reducing or increasing SFK activity experimentally leads to changes in downstream pathways and affects cytoskeletal organization, for example

actin fibers, in other cell types (Thomas et al., 1995; Brandt et al., 2002; Kilarski et al., 2003).

We investigated the consequences of reduced SFK function using *src*$^{-/-}$;*fyn*$^{-/-}$ myotubes, where agrin normally recruited postsynaptic proteins into AChR-containing clusters. This parallels the normal development, until birth, of endplates in *src*$^{-/-}$;*fyn*$^{-/-}$ mice (Smith et al., 2001). But after agrin removal from *src*$^{-/-}$;*fyn*$^{-/-}$ myotubes, clusters of UGC and rapsyn disintegrated in parallel with AChRs. Thus Src and Fyn hold together the postsynaptic apparatus, consistent with AChR pretzel disassembly in Src-AM-expressing myofibers.

SFKs maintain AChR-rapsyn interaction and AChR phosphorylation

In parallel with unstable clusters, AChR-protein interactions are unstable in *src*$^{-/-}$;*fyn*$^{-/-}$ myotubes, and the key compromised interaction is that between AChRs and rapsyn. Agrin-induced increase in AChR-rapsyn interaction correlates highly with clustering (Moransard et al., 2003), and increased rapsyn binding slows metabolic AChR turnover (Gervasio and Phillips, 2005). AChRs and rapsyn are the most abundant postsynaptic components. Occasionally, AChR-rapsyn complexes are linked to the UGC through dystroglycan, giving rise to a corral model in which postsynaptic proteins such as the UGC are held together through many AChR-rapsyn complexes (Apel and Merlie, 1995)(Fig. 10). If the rapsyn-AChR interaction breaks, AChR-UGC interactions and clusters of all postsynaptic proteins are expected to disintegrate, and this is what our data on *src*$^{-/-}$;*fyn*$^{-/-}$ myotubes indeed show. Thus the primary mode of SFK action in postsynaptic stabilization is to maintain the AChR interaction with its anchor rapsyn (Fig. 10, pathway 1).

SFKs also maintain AChR phosphorylation. Phosphorylation of AChR β, required for efficient AChR cytoskeletal linkage and clustering (Borges and Ferns, 2001), can directly be mediated by SFKs, at least in vitro (Swope and Huganir, 1993; Fuhrer and Hall, 1996). Upon agrin stimulation of myotubes, however, SFKs only act in the initial phase, are later compensated by Abl kinases and not necessary for cluster formation (Mittaud et al., 2004). We now find that Src and Fyn are required to maintain β phosphorylation, and this most likely reflects direct phosphorylation. Loss

of β phosphorylation may lead to weaker AChR-cytoskeletal linkage, but we were not able to reliably quantify AChR-extractability after agrin withdrawal due to variation between experiments (R. Willmann and C. Fuhrer, unpublished observations).

In *src*$^{-/-}$;*fyn*$^{-/-}$ myotubes, loss of β phosphorylation after agrin withdrawal is paralleled by loss of AChR-rapsyn interaction. Additional experiments have further corroborated a tight correlation: while agrin induces pronounced β phosphorylation and rapsyn-binding in C2 myotubes, pervanadate treatment causes stronger β phosphorylation and stronger rapsyn binding, revealing a linear relationship between the two events (M. Moransard and C. Fuhrer, unpublished observations). Furthermore, the time course of agrin-induced β phosphorylation exactly parallels that of AChR-rapsyn binding (M. Moransard and C. Fuhrer, unpublished observations). Thus, increased AChR-rapsyn binding may occur through β phosphorylation, via direct protein interaction or through an intermediate linker (Fig. 11, pathway 1). Consequently, loss of β phosphorylation may diminish AChR-rapsyn interaction, causing postsynaptic disassembly. The proposal that rapsyn binds AChRs in several ways - in basal state independent of AChR phosphorylation but after agrin addition through increased receptor phosphorylation (Fig. 11) - is consistent with findings from heterologous cells. Here, rapsyn directly or indirectly interacts with AChRs in multiple ways through association with all receptor subunits (Maimone and Merlie, 1993; Maimone and Enigk, 1999; Bartoli et al., 2001; Huebsch and Maimone, 2003).

More surprisingly, SFKs repress the amount of rapsyn protein (Fig. 11, pathway 2). Correct expression level of rapsyn is important, as its overexpression reduces AChR clustering in myotubes (Yoshihara and Hall, 1993; Han et al., 1999). We did not investigate whether alterations in rapsyn synthesis, degradation or turnover cause the increase in overall rapsyn protein. More importantly, we found that rapsyn's own cytoskeletal link is unaffected by Src and Fyn. In heterologous cells, SFKs form a complex with rapsyn and rapsyn triggers their kinase activity, leading to AChR phosphorylation (Mohamed and Swope, 1999). In myotubes, rapsyn is required for agrin-induced activity of SFKs, implying an interaction between rapsyn and SFKs (Mittaud et al., 2001). Thus SFK and rapsyn seem to be engaged in mutual control, leading to correct rapsyn protein levels and SFK activity. Fine-tuning of such

interactions and activities may be important for correct protein interactions in building up and stabilizing the postsynaptic apparatus, and for appropriate linkage of associated signaling pathways.

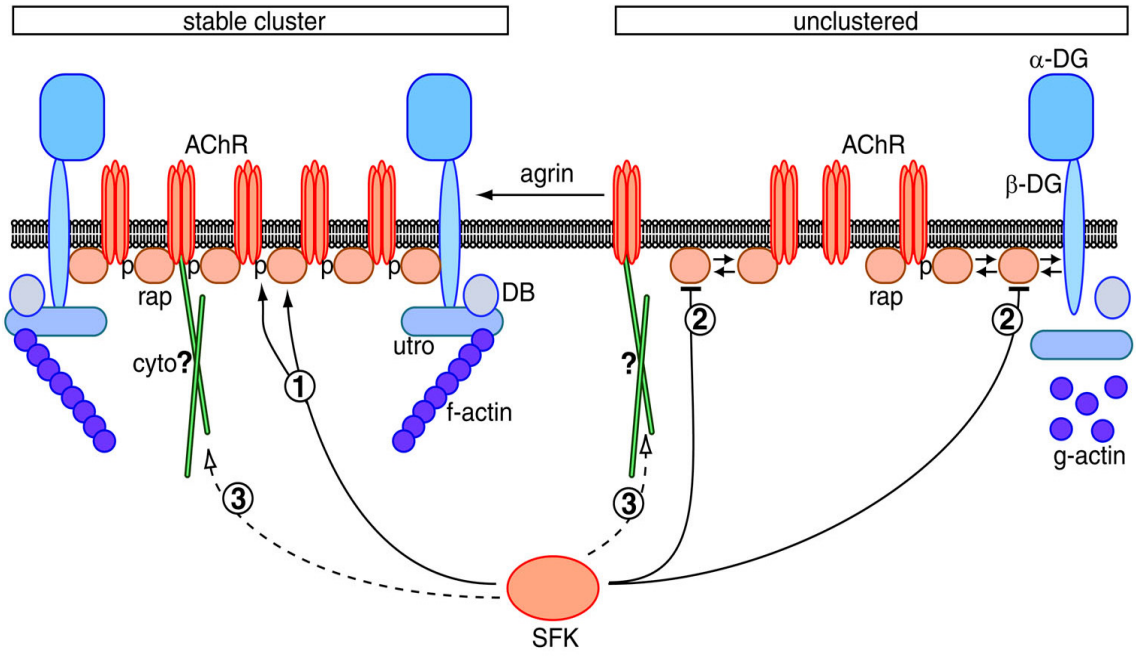


Figure 11. Model of SFK action in stabilization of the postsynaptic apparatus. Unclustered proteins such as rapsyn (Ross et al.) are in equilibrium between free and complexed form, indicated by double arrows. Agrin increases AChR-protein interactions in the process of clustering. SFKs act in postsynaptic stabilization by maintaining the AChR-rapsyn interaction (pathway 1). As rapsyn and AChRs are the most abundant postsynaptic proteins, their interaction plays a core role and holds the postsynaptic apparatus together. This may occur through AChR β phosphorylation (p), which is maintained by SFKs (pathway 1). SFKs also negatively control the overall amount of rapsyn protein (pathway 2). In the absence of Src and Fyn, rapsyn amounts are high and may start saturating binding sites on β-dystroglycan (β-DG) and the AChR, although AChR-dystrobrevin interactions appear normal. SFKs may control another cytoskeletal link (cyto) of the AChR, independent of rapsyn as a linker (hypothetical pathway 3). This would explain the observed weak overall AChR-cytoskeletal linkage in *src*$^{-/-}$;*fyn*$^{-/-}$ myotubes, which is still strengthened upon agrin treatment due to AChR-UGC association. Utro, utrophin; DB, α-dystrobrevin.

SFKs control AChR-cytoskeletal interactions

Unstability of AChR-protein interactions is sufficient to explain the disintegration of postsynaptic protein clusters but may not account for all postsynaptic changes

observed in vivo upon Src-AM expression. Changes in nerve-AChR topology and synaptic nuclei positioning are likely to reflect additional changes in the postsynaptic cytoskeleton as illustrated by loss of synaptic α-tubulin rings. Indeed, cytoskeletal linkage of the AChR is weaker in *src*$^{-/-}$;*fyn*$^{-/-}$ myotubes, yet strengthened by agrin treatment. One candidate mechanism to explain this observation is the UGC and its interaction with F-actin (Winder et al., 1995). We do not think that the higher rapsyn amount in *src*$^{-/-}$;*fyn*$^{-/-}$ cells saturates rapsyn binding sites on AChRs or β-dystroglycan, thereby disturbing AChR-F-actin linkage, since the UGC component dystrobrevin associates normally with AChRs in the mutant cells (Fig. 9A). This leads to the conclusion that another mechanism, potentially involving microtubular organization, accounts for the weaker basal cytoskeletal link of the AChR in *src*$^{-/-}$;*fyn*$^{-/-}$ myotubes. Such a mechanism may not involve rapsyn as a linker, because rapsyn's own cytoskeletal interaction is normal in the mutant cells. More likely, the AChR interacts directly or indirectly with other elements of the cytoskeleton through a novel pathway, before and after agrin treatment, and such linkage depends on Src and Fyn (Fig. 11, putative pathway 3). Agrin-triggered strengthening of the overall AChR-cytoskeletal link may stem from agrin-induced AChR-UGC interaction, as agrin induces normal AChR-dystrobrevin association, in mutant and wild-type myotubes. In such a manner, normal AChR-UGC association in combination with defects in a putative additional cytoskeletal pathway provides an explanation for the observed alteration in overall AChR extractability in SFK-defective cells.

The intermediate elements in the putative SFK-mediated cytoskeletal pathway remain to be identified. Candidates are known SFK-substrates involved in cytoskeleton dynamics, such as cortactin and WASp (both regulating the Arp2/3 complex) (Daly, 2004; Martinez-Quiles et al., 2004) or p190RhoGAP (influencing Rho GTPase activity) (Chang et al., 1995), which all ultimately regulate F-actin assembly. Reduction or increase in SFK activity affects the organization of actin fibers in other cell types (Thomas et al., 1995; Brandt et al., 2002; Kilarski et al., 2003), consistent with our finding that both Src-AM and Src-Y527F expression disassembles AChR pretzels in vivo. Rho, along with Rac and Cdc42, is already known to play a role in AChR cluster formation in cultured myotubes (Weston et al.,

2000; Weston et al., 2003). SFKs also influence the tubulin network (Cox and Maness, 1993), and we have observed changes in synaptic tubulin organization following Src-AM expression.

It remains to be investigated what other connections between SFKs and the cytoskeleton and its regulators exist at the NMJ, affecting postsynaptic stability. As balanced SFK activity is important (Fig. 1-3), SFKs may be counteracted by tyrosine phosphatases. Phosphatase activity dissolves AChR hot spots in cultured Xenopus myocytes, and some of this activity is triggered by agrin application (Madhavan et al., 2005). The phosphatase Shp-2 is a possible candidate, as blocking Shp-2 increases spontaneous AChR clustering (Madhavan et al., 2005). The balance between SFKs and phosphatases offers a fine-tuning system to shape the postsynapse. It will be interesting to assess the role of such a system in vivo, also in the first weeks of postnatal NMJ development, when synapse elimination occurs. In this process, AChR regions can be selectively destabilized paralleled by nerve withdrawal (Lichtman and Colman, 2000), and a tyrosine kinase-phosphatase regulation is an attractive candidate mechanism.

Materials and Methods

Src constructs, electroporation, whole mount preparation and immunohistochemistry. Three different mutant Src expression constructs, each containing a CMV promoter, and an empty pLNCX vector as a control were used for electroporation into the soleus muscle of mice. Src-AM (Kaplan et al., 1994) was kindly provided by Dr. Pam Schwartzberg (NIH). Src-K295M (Mohamed et al., 2001) was received from Dr. Sheridan Swope (Georgetown University, Washington DC), and Src-Y527F from Dr. Joan Brugge (Harvard Medical School, Boston). Mutant Src constructs (8 μg/μl) and GFP containing a nuclear localization signal (NLS-GFP; 4 μg/μl) were mixed to yield final DNA concentrations of 2:1 (Src:GFP), and were first injected extrasynaptically into the soleus muscle of adult C57BL/6 mice (>6 months). Legs were then closed, electrodes were pressed against the limb, and electroporation was performed as described previously (Kong et al., 2004) using an ECM 830 electroporation system (BTX, Holliston, MA). Eight pulses of 20 ms were applied at a

frequency of 1 Hz with voltage set to 200 V/cm. At the site of DNA injection, electroporation efficiency is highest, and once DNA constructs have entered muscle fibers, they diffuse within those to regions including the synapse (Kong et al., 2004). The electroporated muscles were analyzed after 6 weeks. Muscles were dissected and injected with 2% para-formaldehyde solution for fixation. This treatment is optimal for whole-mount analysis, as it swells the muscle, widening gaps between individual muscle fibers, facilitating further fiber dissection. Tissue was placed in 2% para-formaldehyde solution, then overnight in 10% sucrose at 4°C, and teased into thin fiber bundles of 5-10 myofibers. Whole mount preparations were triple-labeled as described earlier (Kong et al., 2004). Briefly, AChRs were stained to visualize NMJs using rhodamine-coupled α-bungarotoxin. A mixture of rabbit polyclonal antibodies against neurofilament (Sigma, USA) and synaptophysin (Dakocytomation, Denmark), followed by Cy5-conjugated goat anti-rabbit or Alexa 350 goat anti-rabbit antibodies, was used to visualize motorneurons and nerve endings. The green channel was reserved for GFP. For cytoskeletal stainings, Alexa-350-coupled phalloidin (Molecular Probes, Eugene, USA) was used to label F-actin. Monoclonal anti-α-tubulin antibodies (clone DM1A from Sigma, Missouri, USA), followed by Cy5-conjugated goat anti-mouse antibodies, were used to visualize synaptic tubulin rings beneath the endplate. Conventional fluorescence imaging was done using a Zeiss Axioskop 2 microscope equipped with a Hamamatsu Orcacam digital camera.

Immunohistochemistry on cross-sections. Soleus muscle tissue prepared and fixed for whole-mount analysis (see above) was embedded into O.C.T. (Tissue-Tek; Sakura Finetek, Zoeterwoude, NL) and frozen in a cooling chamber (maintained at -4°C with surrounding liquid nitrogen) containing isopentane (Fluka, Germany) for approximately 5 minutes. Muscles were then cut into 14 μm cryosections (at -18°C). Sections were stained with rhodamine-coupled α-BT to visualize the AChRs at the NMJ. GFP signals were enhanced with polyclonal anti-GFP antibody (Molecular probes, Eugene, Oregon, USA) followed by Alexa 488-coupled goat anti-rabbit antibodies. Nuclei were visualized by DAPI (Hoechst nuclear stain; Molecular Probes).

The fixation procedure, optimized for whole mount analysis, expanded gaps between individual myofibers, rounding off some fibers and allowing entry of other cells (see Fig. 4C, D). Nonetheless, we could unequivocally identify muscle fibers (due to diffuse GFP-signal) and the positioning of nuclei within those fibers (as most of the strong nuclear GFP signals overlapped with DAPI).

Confocal microscopy, imaging and quantitation. Confocal laser scanning microscopy was performed using an inverted Leica confocal SP2 microscope coupled to a Silicon Graphics Workstation. A minimum of 40 stacks per image, each section at approximately 0.3 μm thickness, were taken. These confocal stacks were shown as a maximal projection and eventually imported into a 3D image processing software, Imaris 4.1.1, for image reconstruction and rotation (Fig. 1-4). To quantify disassembly, synaptic contacts as visualized by the postsynaptic AChRs and the presynaptic nerve terminal were scored based on size and degree of disassembly. Intact endplates were large pretzel shaped structures (25 - 40 μm) and continuous along their contours. A partial disassembly was scored when the endplate was broken up into more than 2 main fragments and was porous. In such cases it was still possible to identify the parts of a pretzel. In a complete disassembly the structures completely dissolved into several fragments, which were less than 5 μm in length. These were scored as synaptic sites because of the nerve staining and surrounding intact endplates in GFP-negative fibers. Statistics were performed by averaging 3 sets of independent electroporation experiments, with a minimum of 30 pictures taken for each condition. In all cases the NLS-GFP signal colocalized with the AChR stain and lay beneath the endplates, suggesting that the electroporation was specific to the muscle nuclei and was not an indirect effect due to electroporation of Schwann cells. Apart from synaptic muscle nuclei, also extrasynaptic nuclei were GFP-positive in most cases.

Cell culture and agrin treatment. *src*$^{-/-}$*;fyn*$^{-/-}$ cells (clones DM15 and DM11) and their corresponding wild-type cells (clones SW10 and SW5) were grown as described earlier (Smith et al., 2001), in DMEM supplemented with 2 mM glutamine, 10% fetal bovine serum, 10% horse serum, 2% chick embryo extract, penicillin-streptomycin, and 20 U/ml recombinant mouse interferon-γ. In all assays we tested both mutant and

both wild-type clones, also in pairwise comparison. There were no differences between clones of the same genotype, excluding the possibility of clonal variation.

For biochemical analysis, cells were plated on matrigel-coated tissue culture dishes (Nunc, Life Technologies, Basel, Switzerland) at 0.18 Mio/10 cm dish (wild-type) and 0.35 Mio/10 cm dish (mutants). For immunocytochemistry, wild-type and mutant cells were plated on matrigel-coated chamber slides (Nunc) at 0.02 Mio/2.4 ml and 0.04 Mio/2.4 ml, respectively. Myoblasts were grown to 80% confluency at 33ºC and 5% CO_2 and cells were shifted to fusion media (DMEM supplemented with 2 mM glutamine, 10% fetal bovine serum, 10% horse serum, 2% chick embryo extract and penicillin-streptomycin) at 39ºC, 10% CO_2 to differentiate into myotubes. Both growth and fusion media were replaced every day to ensure good myotube morphology and experiments were conducted on mature myotubes. To induce clustering, myotubes were incubated with 0.5 nM recombinant neural agrin for 15-20 hours (C-$Ag_{12,4,8}$) (Fuhrer et al., 1997). To analyze stability of agrin-induced clusters, myotubes, following overnight agrin treatment, were subsequently washed twice with fusion media and maintained in differentiation media lacking agrin for 3-6 hours. This procedure was shown to be efficient in removing the vast majority of agrin from cells (Mittaud et al., 2004). As shown before (Smith et al., 2001), disassembly of AChR clusters occurs in *src*$^{-/-}$;*fyn*$^{-/-}$ myotubes already after 3 hours of agrin withdrawal and more evidently after 5 hours. In parallel wild-type or C2C12 myotubes, little disassembly is visible under these conditions.

AChR precipitation assays and immunoblotting. To examine the association of postsynaptic proteins with the AChR, myotubes were rinsed with ice cold PBS containing 1 mM Na-orthovanadate and 50 mM NaF and extracted at 4ºC in lysis buffer. The lysis buffer contained 1% NP-40 and an excess of protease and phosphatase inhibitors as described earlier (Fuhrer et al., 1999). Lysates were processed and AChRs precipitated using biotinylated α-BT as detailed before (Tox-P) (Mittaud et al., 2001). As a control, an excess of free α-BT (+T) was added to some lysates to judge the specificity of protein association with the AChR. Precipitates were subjected to SDS-PAGE and immunoblotting.

To quantify proteins in total cell extracts, parts of lysates were processed, without precipitation, in parallel by SDS-PAGE and immunoblotting. To ensure proper loading, protein estimations of total lysates were done prior to loading using a standard BCA protein assay kit (Pierce), and amounts of each protein were normalized to the β-subunit of the AChR on the immunoblot.

In Western blots, α-dystrobrevin-2 was visualized using rabbit polyclonal β1CT-FP antibodies (gift from Dr. Derek J. Blake; University of Oxford). Conditions and antibodies to detect rapsyn, phosphotyrosine, AChR α and β subunit, utrophin and MuSK were as detailed before (Marangi et al., 2001; Moransard et al., 2003). Quantitations of the immunoblots were done by scanning exposed films containing gray, nonsaturated signals with a computerized densitometer (Nikon Scantouch 210) and using the NIH Image J 1.29X software (National Institute of Health, USA). Experiments were repeated at least 5 times to obtain consistent results.

AChR extractability assay. We used a modified version of a sequential extraction procedure detailed before (Borges and Ferns, 2001; Moransard et al., 2003). All steps were carried out on ice if not else specified. 2-3 days old myotubes grown in 10-cm dishes were washed briefly with ice-cold PBS (+ 1 mM Na-Orthovanadate) before adding 1 ml of lysis buffer (30 mM triethanolamine pH 7.5, 50 mM NaCl, 5 mM EDTA, 5 mM EGTA, 50 mM NaF, 1 mM Na-Orthovanadate, benzamidine, NEM, Na-tetrathionate, 50 μM phenylarsine oxide, 10 mM pNpp, 25 μg/ml aprotinin and leupeptin, 1 mM PMSF) containing a final concentration of Triton X-100 ranging from 0.03% to 0.09%, according to the conditions tested. The exact time for the first extraction was 21 minutes; a timer was started just after addition of lysis buffer to the first plate. Cells were scraped from each plate and the extracts were homogenized by pipetting up and down 10x, then transferred to tubes that were rotated for the rest of the time at 4°C. Extracts were centrifuged for 3 min at 14000 rpm and 4°C in a table eppendorf centrifuge, supernatants were transferred to fresh tubes and called „first extraction“. Pellets were resuspended in 1 ml lysis buffer containing 1% Triton X-100 for a total extraction time of exactly 15 min. Again, timer was started after the first resuspension and tubes were rotated together at 4°C for the rest of the time, and finally centrifuged under the same conditions. Supernatants were transferred to fresh

tubes and called „second extraction“. Both series of tubes were then subjected to AChR-precipitation with biotinylated α-BT, followed by immunoblotting for AChR α as described above.

Immunocytochemical staining procedures and quantitation of clusters. Cultured myotubes were grown in matrigel-coated multiwell chamber slides. Stainings for AChR, α-dystrobrevin-1, utrophin, rapsyn, α-dystroglycan, syntrophin isoforms and phosphotyrosine were all done exactly as described before (Marangi et al., 2001; Moransard et al., 2003). Myotubes were examined at 400-fold magnification in both Rhodamine and Fluroscein channels with a fluorescence microscope (Axioskop II, Zeiss, Germany). Representative pictures were taken and processed with a cooled digital Camera (Orcacam, Hamamatsu, Japan).

To quantitate clusters, a total of 20 representative pictures per condition were taken from several chamberslides. Clusters of AChR and the respective postsynaptic proteins were counted based on signal intensity (clearly distinguishable from the diffuse background) and the length of the cluster being at least 10 μm. Numbers of clusters per myotubes were calculated independently for the AChR and the postsynaptic marker and averaged. Co-localization of clusters were calculated as the percentage of number of AChR clusters containing the postsynaptic marker protein. Staining experiments were repeated several times to ensure reproducibility.

Chapter 3

The text and figures in this chapter represent a manuscript in preparation.

Kinase signaling in cytoskeletal remodelling and AChR clustering

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Acknowledgements: We thank Dr. Mathias Höchli and Dr. Anne Greet Bittermann from the Laboratory of Electron Microscopy at the University of Zürich for their excellent technical assistance with the confocal microscope. This work was supported by the Eric Slack-Gyr Foundation, and by grants from the Swiss National Science Foundation, the Swiss Foundation for Research on Muscle Diseases and the Zürich Neuroscience Center (to C.F.).

Abstract

High density accumulation of AChRs, a hallmark step in synaptogenesis, is achieved by coordinated action of F-actin assembly and tyrosine phosphorylation of crucial postsynaptic proteins during development; but how these two processes are regulated at the NMJ remains poorly characterized. Our earlier studies suggested that balanced activity of SFKs is critical in maintaining the NMJs in vivo and in vitro. Changes in SFK activity led to abnormalities in nerve terminals, synaptic nuclei positioning and disruption of the endplate as a consequence of decreased protein interactions and tyrosine phosphorylation of the AChRs. Furthermore, loss of sub-synaptic α-tubulin rings was observed at adult NMJs. Most importantly the link of the AChRs to the cytoskeleton was weak in cultured *src*$^{-/-}$*;fyn*$^{-/-}$ myotubes. In fibroblasts, F-actin polymerization is known to be regulated by key cytoskeletal intermediates like cortactin and p190RhoGAP through changes in their tyrosine phosphorylation content. Therefore we investigated if both cortactin and p190RhoGAP are SFKs substrates in muscle cells, and whether changes in their tyrosine phosphorylation content occur in *src*$^{-/-}$*;fyn*$^{-/-}$ myotubes, possibly explaining the instability of AChR clusters observed in these mutant cells. Our results from pharmacological inhibitor studies show that cortactin and p190RhoGAP are indeed SFK substrates and that the tyrosine phosphorylation content of cortactin at residues PY421 and PY466, and the overall tyrosine phosphorylation of p190RhoGAP are reduced significantly in the *src*$^{-/-}$*;fyn*$^{-/-}$ myotubes under steady-state conditions. In addition, cortactin and p190RhoGAP were formed as sub-synaptic rings beneath the endplate at mature NMJ. These studies suggest that SFK regulation of cytoskeletal intermediates like cortactin and p190RhoGAP could have important consequences in the F-actin-mediated AChR clustering and maintenance at the NMJ.

Introduction

High density accumulation of acetylcholine receptors (AChRs) in the muscle is a hallmark step in the development of the vertebrate neuromuscular junction (NMJ). This is achieved by innervation, regulated signaling orchestrated by tyrosine kinases and phosphatases, and eventually a link of receptors to the underlying cytoskeleton.

Much is known about the signal transduction mechanism involved in the clustering of AChRs at the NMJ. Agrin, a heparin-sulfate proteoglycan, is released by the motor neuron and activates a muscle-specific receptor tyrosine kinase (MuSK), which eventually leads to the clustering of AChRs in a rapsyn-dependent manner (Gautam et al., 1995; Bezakova and Ruegg, 2003; Luo et al., 2003). Crucial in this signaling pathway is the phosphorylation of MuSK and AChR β and δ subunits; receptor phosphorylation links the AChR to the cytoskeleton (Wallace, 1992; Ferns et al., 1996; Mittaud et al., 2001). Blocking tyrosine phosphorylation (Wallace, 1995; Ferns et al., 1996; Mittaud et al., 2001) or cytoskeletal assembly by preventing F-actin polymerization with inhibitors strongly interferes with clustering of AChRs (Dai et al., 2000). Thus tyrosine phosphorylation and F-actin assembly are a requirement for AChR clustering and may serve as a scaffold for the assembly of signalling machineries.

Although several studies showed independently that tyrosine phosphorylation and F-actin assembly are important in AChR clustering, the exact mechanism by which the two processes co-operate is not clear. Recent discovery of several MuSK effectors involved in actin cytoskeletal oganization has shed some light on the molecular events downstram of agrin-induced MuSK activation. Cytoplasmic effectors like Abl kinases, GGT (geranylgeranyltransferase 1) and Dvl (dishevelled-1) interact with MuSK and the kinase activate Rac, Rho, Cdc42 and PAK1 leading to actin cytoskeletal reorganization and AChR clustering, likely through the tumor suppressor protein APC (Adenomatous polposis Coli), an actin binding element (Luo et al., 2002; Pendergast, 2002; Luo et al., 2003; Wang et al., 2003). In addition cortactin is localized at spontaneous and growth factor coated bead- induced AChR clusters in

Xenopus muscle cells (Peng et al., 1997). Evidence from our earlier studies showed that Src-family kinases (SFKs) are important in maintaining AChR-cytoskeletal interactions (Sadasivam et al., 2005). Changes in the SFK activity lead to destabilization of AChR-protein interactions both at the NMJ and in cultured myotubes. Alterations observed in vivo after expression of a kinase-inactive Src construct affect the nerve-AChR cluster topology, the positioning of the synaptic myonuclei and the organization of α-tubulin subsynaptic rings (Sadasivam et al., 2005). In addition, in cultured *src*$^{-/-}$*;fyn*$^{-/-}$ myotubes the basal cytoskeletal link of the AChRs is weak although strengthened by agrin. All these observed changes imply a weak cytoskeletal link and a SFK-dependent mechanism of maintenance of normal AChR-cytoskeletal interactions.

In order to investigate the intermediate elements in the putative-SFK mediated cytoskeletal pathway we studied several downstream candidates involved in F-actin assembly. We particularly looked at cortactin, an important F-actin binding protein known to co-localize at spontaneous and bead-induced AChR clusters in Xenopus muscle (Peng et al., 1997; Dai et al., 2000). We also examined p190RhoGAP, which regulates the activity of the Rho family of small GTPases, which in turn affect F-actin assembly (Chang et al., 1995). Our studies indicate that cortactin and p190RhoGAP are indeed Src substrates in cultured C2C12 myotubes, based on inhibitor experiments, and that they are enriched at sub-synaptic sites underneath the AChR clusters at the NMJ in vivo. The basal level of tyrosine phosphorylation of cortactin at residues PY421 and PY466 and of p190RhoGAP is greatly reduced in *src*$^{-/-}$*;fyn*$^{-/-}$ myotubes suggesting an early defect in the cytoskeletal pathway that stabilizes AChR clusters.

Results

Cortactin and p190RhoGAP are SFK substrates and not induced in the agrin signaling pathway

We first examined cortactin, an important nucleation promoting factor (NPF) of Arp2/3-mediated F-actin assembly (Kaksonen et al., 2000). We analysed whether cortactin is a SFK substrate in myotube cultures, which might be important in

regulating F-actin assembly during synapse formation. For this purpose we used C2C12 myotubes and treated them with inhibitors of SFKs. We used three specific inhibitors - PP1, PP2 and SU6656 (Blake et al., 2000; Smith et al., 2001) - to be sure that any observed effects indeed originate from specific SFK inhibition. Myotubes were treated overnight with 10 μM of the inhibitors and re-treated the next day for 90 minutes before the start of a precipitation assay. The cells were lysed using a strong detergent mix like RIPA buffer to solubilize as much cortactin as possible. Following lysis, cortactin was immunoprecipitated and subjected to immunoblot analysis using specific phospho-cortactin antibodies PY421 and PY466 (for details see Materials and Methods). The two phosphotyrosine residues on cortactin (PY421 and PY466) have been proposed as SFK substrates and in regulating F-actin assembly in other cell systems (Wu and Parsons, 1993; Fan et al., 2004).

Our results show that inhibitor treatments with PP1, PP2 and SU6656 reduce cortactin phosphorylation levels at residues PY421 and PY466 by about 70% and 50% respectively, when compared to the controls (Figure 1 A+B). In order to investigate if the tyrosine phosphorylation at these residues is agrin-induced, we treated myotube cultures with 1 nM or 5 nM agrin overnight and analysed phosphorylation. We did not observe any detectable change between the no-agrin and agrin-treated conditions (data not shown). Our data suggest that basal phosphorylation of cortactin is dependent on SFK activity, is not induced by agrin, and may be a step in synapse development or maintenance.

Additionally we looked at another cytoskeletal intermediate, p190RhoGAP, which is upstream of F-actin polymerization and downstream of Src in other cell systems. p190RhoGAP has been implicated in regulating Rho GTPase activity-mediated F-actin assembly (Chang *et al*., 1995). Therefore we examined if RhoGAP is a Src substrate in muscle using the same inhibitor treatments as before. The cells were lysed and p190RhoGAP was immunoprecipitated followed by an immunoblot using anti-phosphotyrosine antibodies.

Our results suggest that the overall phosphorylation of p190RhoGAP is significantly decreased by 60-70% in the presence of SFK inhibitors. Like cortactin, p190RhoGAP phosphorylation also does not show a response to agrin treatment (data

not shown). Taken together, the results introduce cortactin and p190RhoGAP as SFK-substrates in muscle.

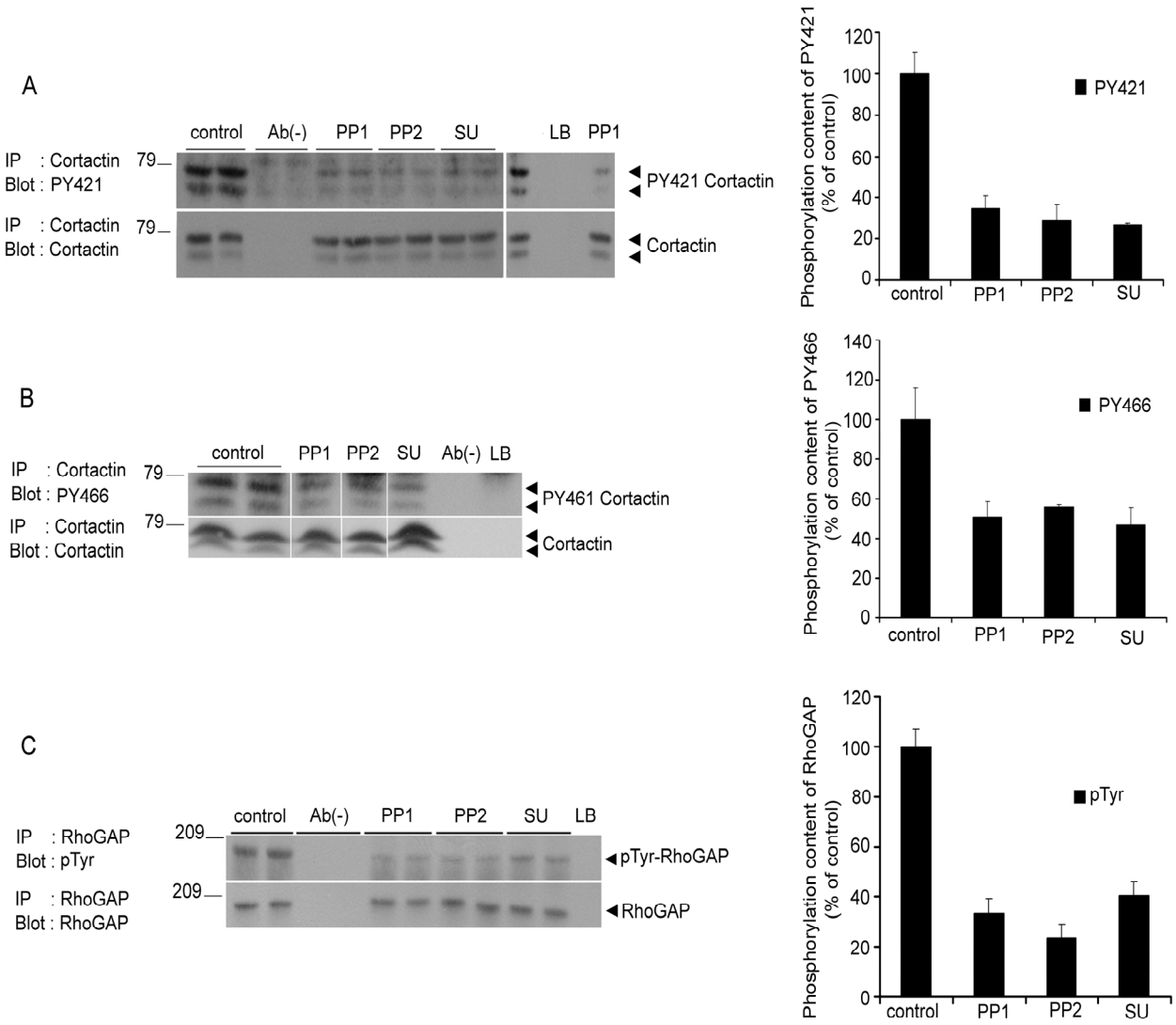

Figure 1: Cortactin and RhoGAP are SFK substrates in myotubes. C2C12 cells were treated overnight with 10 µM PP1, PP2 and SU6656 as indicated and re-treated the next day before the precipitation assay. Cortactin and RhoGAP were precipitated (IP) from the lysates using monoclonal anti-cortactin 4F11 and anti-RhoGAP (p190) respectively. The associated tyrosine phosphorylation was detected by Western blotting. As controls, lysates without antibody [Ab(-)] and lysis buffer with only the antibody (LB) were included during the precipitation assay. In the bottom parts, 30% of the immunoprecipitate were re-loaded and blotted with the same primary antibody used for precipitation, to ensure that the amounts of protein precipitated were the same in all the lanes. **A** and **B** represent the phosphorylation on tyrosine residue PY421 and PY466 of cortactin, respectively. Cortactin is known to migrate as a doublet band with apparent molecular weight of 70-75 KD. The level of phosphorylation upon inhibitor

treatment is decreased by 50-70% when compared to the controls. **C** represents the overall tyrosine phosphorylation associated with RhoGAP. Its phosphorylation also decreases greatly on inhibitor treatment. Data are sometimes shown as duplicate lanes. The respective graphs, obtained from densitometric scanning, quantitate the percentage of tyrosine phosphorylation associated with the precipitated protein, the controls are the untreated samples and set to 100%. Data represent mean ± SEM of at least 3 experiments.

Reduced phosphorylation of cortactin and p190RhoGAP in src$^{-/-}$;fyn$^{-/-}$ myotubes compared to wild-type controls

In our earlier studies (Sadasivam et al., 2005) *src$^{-/-}$;fyn$^{-/-}$* myotubes showed unstable clusters of AChRs and other postsynaptic proteins after agrin withdrawal. Biochemical studies also indicated reduced AChR-protein interactions after agrin withdrawal. Most importantly, the overall basal cytoskeletal link of the AChR was weak in *src$^{-/-}$;fyn$^{-/-}$* myotubes although strengthened by agrin. Consistently with this, we know find that cortactin and p190RhoGAP require SFK activity for their basal phosphorylation independent of the agrin signaling pathway (Figure 1).

Therefore we investigated if the changes observed in the *src$^{-/-}$;fyn$^{-/-}$* myotubes could be linked to decreased phosphorylations of cortactin (PY421 and PY466) and p190RhoGAP. For this purpose, cortactin and p190RhoGAP were precipitated from wild-type and mutant myotubes and analysed by immunoblots using specific antibodies against phosphotyrosine as described before.

Our results indicate an overall decrease in the basal phosphorylation of cortactin (at residues PY421, PY466) and of p190RhoGAP in *src$^{-/-}$;fyn$^{-/-}$* myotubes compared to wild-type cultures. Defects in phosphorylation of these two cytoskeletal intermediates important in F-actin assembly observed in *src$^{-/-}$;fyn$^{-/-}$* myotubes may partly explain the AChR instability phenotype in these mutants.

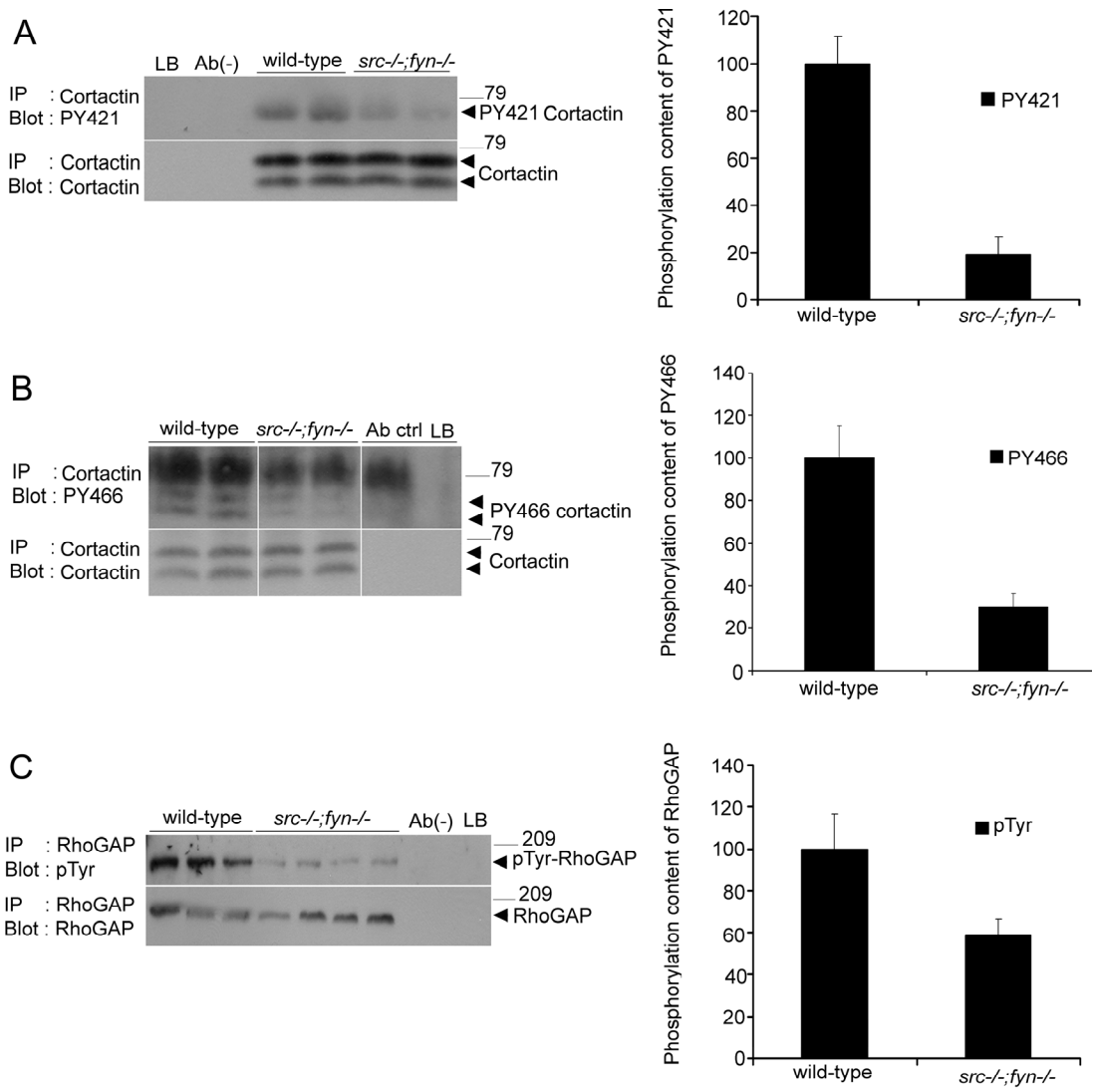

Figure 2: In *src*$^{-/-}$;*fyn*$^{-/-}$ myotubes tyrosine phosphorylation of cortactin and p190RhoGAP is reduced. Cells were lysed, and cortactin or p190RhoGAP were immunoprecipitated, including controls as described in Figure 1. **A,B** and **C** indicate the amount of tyrosine phosphorylation associated with cortactin (PY421), cortactin (PY466) and p190RhoGAP, respectively. In **A** and **B**, the amounts of proteins precipitated are the same in all the lanes as shown in the bottom part of the blots. In **A**, the PY421 signal corresponds to the major upper band of the cortactin blot. In **C**, some variation was observed as shown in the multiple lanes for wild-type and src$^{-/-}$;fyn$^{-/-}$ cells. Graphs indicate that the level of phosphorylation is reduced by 40 to 80% in *src*$^{-/-}$;*fyn*$^{-/-}$ cells as compared to the wild-type controls set to 100%. Data were obtained by dividing the phosphorylation signals through the amount of protein and represent mean ± SEM of at least 5 experiments.

Cortactin and p190RhoGAP localize at the NMJ in association with AChR clusters

We next wanted to see if cortactin and RhoGAP localized at mature synapses in vivo, associated with the AChR clusters. For this purpose, fibers were isolated from the soleus muscle of adult mice and stained for postsynaptic structures with rhodamine-coupled α-bungarotoxin (against AChRs; to visualize the endplate). The presynaptic nerve terminal was stained with a mixture of antibodies against neurofilament (NF) and synaptophysin (Syn) (see Materials and Methods). In triple or double stainings, cortactin or p190RhoGAP were assayed by antibody staining.

We often observed that in mature synapses, the AChR clusters are pretzel shaped, about 20 μM in diameter, with the synaptic nuclei lying beneath and the nerve terminal above. A confocal three-dimensional imaging using Imaris software further illustrated the relative positioning of the nerve, endplate and the synaptic nuclei (Sadasivam et al., 2005).

In the present experiments we find that both cortactin and p190RhoGAP offen localized as sub-synaptic rings beneath the AChR clusters. Although both cortactin and p190RhoGAP antibodies also stained additional structures like the nerve and probably connective tissue or blood vessels, it was possible using 3D reconstruction studies in the case of cortactin, to specifically look at sub-synaptic sites by rotating the images around the X-axis (Figure 3). Additionally the staining of the nerve helped to subtract nerve-derived information for the two proteins. For p190RhoGAP we identified ring stainings (Fig 3C), but the subsynaptic localization remains to be determined by confocal 3D reconstruction.

Sub-synaptic rings have been implicated before for α-tubulin, often found around synaptic nuclei at the NMJ (Ralston et al., 1999). Additionally these synaptic α-tubulin rings dissolved when a kinase-inactive Src construct (Src-AM) was electroporated into the soleus muscle of adult mice, suggesting an SFK dependent maintenance of α-tubulin at the NMJ (Sadasivam et al., 2005). It would be interesting to also investigate if cortactin and p190RhoGAP also require SFK activity to maintain their distribution in vivo.

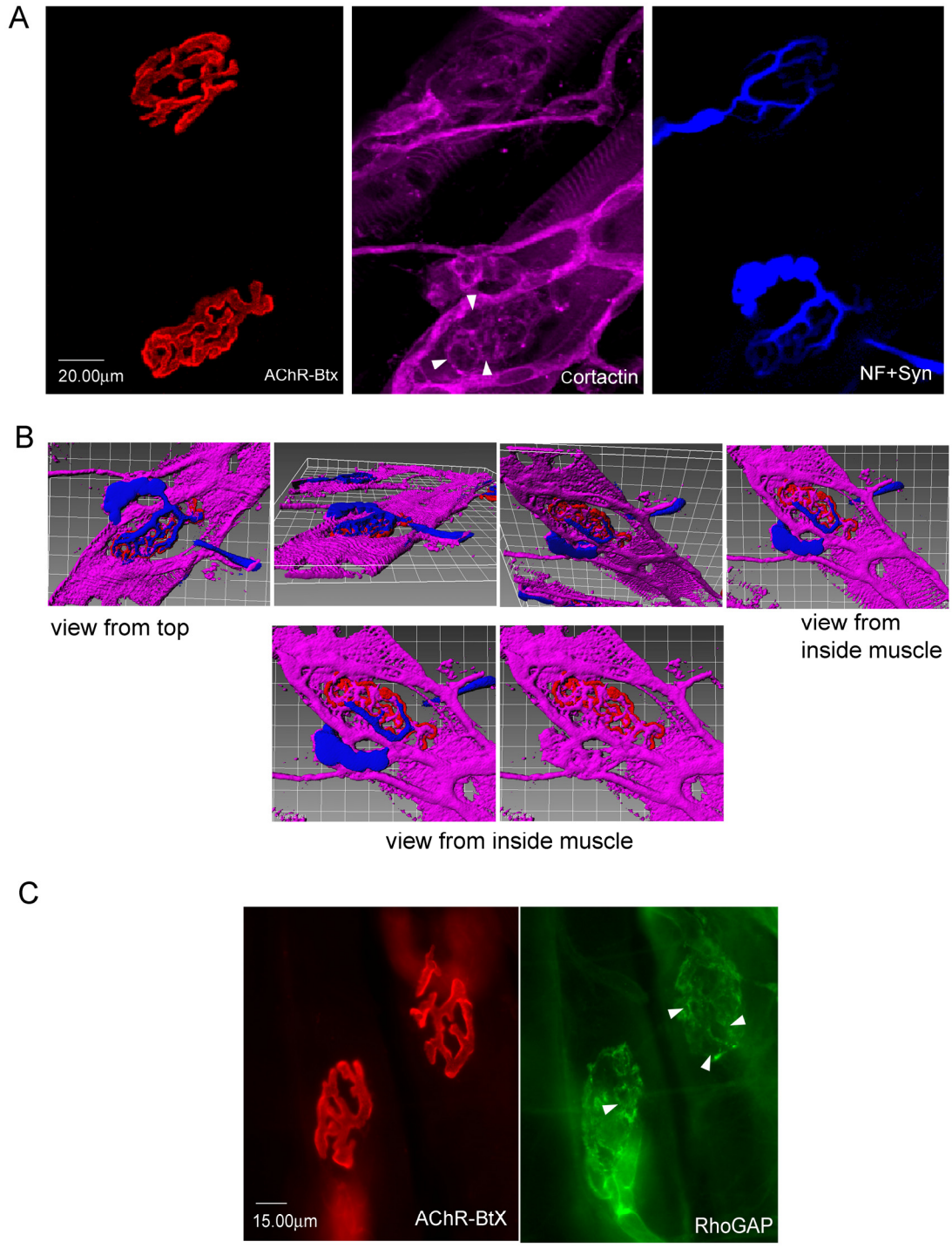

Figure 3: Cortactin and p190RhoGAP localize at the NMJ in vivo. Soleus muscles of adult mice were dissected and whole mounts of fibres were stained with α-BT-rhodamine (Parker et al.) and a mixture of neurofilament (NF) and synaptophysin (Syn) antibodies (in blue). For cortactin and p190RhoGAP stainings anti-cortactin 4F11 and RhoGAP (p190) monoclonal antibodies were used followed by Cy5-

conjugated or Alexa 488 conjugated goat anti-mouse secondary antibodies (shown in purple or green). **A**, Confocal microscopy shows cortactin as subsynaptic rings at the NMJ (arrows), associated with the AChRs and the nerve. Although cortactin antibody stains additional structures like the nerve and connective tissue, a 3D reconstruction using Imaris 4.1.1 (**B**) allows rotation around the X-axis to illustrate better the synaptic cortactin rings beneath the endplate, also shown in higher magnification with and without the nerve staining. The nerve staining also helped to subtract nerve derived information of cortactin stains. **C**, conventional microscopy indicating p190RhoGAP (in green) localizing with the AChRs (in red) at the NMJs in vivo. Rings are visible (arrows) and are most likely subsynaptic. The exact topological organization in relation to AChR clusters remains to be analysed by confocal microscopy.

Discussion

Our data reveal that both cortactin and p190RhoGAP are substrates of SFKs in myotube cultures and that *src*$^{-/-}$*;fyn*$^{-/-}$ myotubes show overall reduced basal phosphorylation of these two proteins. However the tyrosine-phosphorylation of the two proteins is not induced by agrin and may be a step in postnatal stability and maintenance. Additionally, we see that both cortactin and p190RhoGAP localize as synaptic rings at mature synapses beneath the endplate in vivo. For cortactin, the subsynaptic localization of these rings has been confirmed by confocal microscopy.

Interference with SFK functions using inhibitors proved that both cortactin and p190RhoGAP are SFK substrates in muscle cultures. Although functional roles of tyrosine phosphorylation on these cytoskeletal intermediates have been shown before in other cell systems, the role of cortactin and p190RhoGAP at the NMJ is not known. Cortactin is known to localize at spontaneous and bead-induced AChR clusters in Xenopus muscle cells (Peng et al., 1997; Dai et al., 2000) but the functional implications of such localization are not clear. Our current understanding that cortactin and p190RhoGAP are SFK substrates in muscle is interesting because studies in other systems have shown that levels of tyrosine phosphorylation on such proteins regulate F-actin assembly and stability. For instance, inhibitors of F-actin assembly like Latriculin-B are known to cause an increased phosphorylation of cortactin on tyrosine residues (Fan *et al*., 2004). Also, tyrosine phosphorylation of p190RhoGAP is known to regulate its binding with a negative regulatory protein, p120RasGAP (Chang et al., 1995). The interaction between the two proteins regulates

GAP functions on Rho and Rac activation, which in turn affects F-actin assembly (Chang et al., 1995). Interestingly, co-operation between Rho and Rac along with another member of the family of small GTPases, Cdc42, is important in the formation of AChR clusters in muscle cells (Weston et al., 2000; Weston et al., 2003). Taken together all these findings suggest that SFKs regulate F-assembly, and our finding that cortactin and p190RhoGAP are SFK substrates has significant implications in synapse formation and development.

We find that *src*$^{-/-}$*;fyn*$^{-/-}$ myotubes show overall decreased basal phosphorylation of cortactin and p190RhoGAP. This is an interesting observation because it is consistent with the overall weak basal cytoskeletal link of the AChRs in these mutant cell lines (Sadasivam et al., 2005). *src*$^{-/-}$*;fyn*$^{-/-}$ myotubes in general shown an AChR-instability phenotype as discussed in Chapter 2. There is a paralleled destabilization of AChR clusters along with other postsynaptic protein clusters after agrin withdrawal. Also, AChR-protein interactions rapidly decrease after agrin withdrawal. All these changes could arise from a common defective mechanism, which might be the cytoskeleton or defective phosphorylation of crucial proteins important for signaling and cytoskeletal anchoring. Although agrin seems to rescue or compensate for the instability phenotype seen in *src*$^{-/-}$*;fyn*$^{-/-}$ myotubes, basic processes underlying synapse development are defective in the mutants as indicated in our studies.

Agrin did not seem to have an effect on the phosphorylation of cortactin or p190RhoGAP. It could be that SFK action in regulating F-actin assembly is required much before the initiation of agrin-induced clustering or that this action is a process independent of the agrin signaling pathway. More likely, cortactin or p190RhoGAP phosphorylation may play a role in cluster stabilization postnatally, independent of regulation by agrin. Indeed, agrin might be required only for certain processes of synapse development like formation of receptor clusters. Once such clusters are formed, different mechanisms are involved in their maintenance (Willmann and Fuhrer, 2002). One such mechanism is SFK-mediated tyrosine phosphorylation and fine tuning of protein interactions (Sadasivam et al., 2005).

We also find that cortactin and p190RhoGAP localize at mature NMJs in vivo. They appear similar to α-tubulin rings found around synaptic nuclei beneath the

endplate (Sadasivam et al., 2005). This confirms and extends previous studies on cortactin localization with respect to AChR clusters in Xenopus muscle cells (Peng et al., 1997). It remains to be identified if SFK action on cortactin and p190RhoGAP is required for stabilization of NMJs in vivo. For this purpose electroporation of mutant Src constructs like kinase-inactive Src (Sadasivam et al., 2005) could confirm a SFK role in cytoskeletal maintenance. The localization of cytoskeletal proteins like cortactin, p190RhoGAP and α-tubulin at mature synapses could have implications in AChR turnover and in the transport of newly synthesized receptors from the synaptic nuclei to the membrane surface. Therefore the maintenance of such structures by SFKs could be of significance in the development and stabilization of the NMJ.

Materials and Methods

Cell cultures: C2 (C2C12), *src*$^{-/-}$*;fyn*$^{-/-}$ (clone DM15) and their corresponding wild-type cells (clone SW10) were propagated and fused to form myotubes as described earlier (Smith et al., 2001; Marangi et al., 2002). Cells were treated with the neural form of agrin (C-Ag12,4,8) derived after transfecting COS cells with constructs encoding the C-terminal half of neural agrin as described earlier (Fuhrer et al., 1997). To inhibit Src tyrosine kinases, C2 myotubes were preincubated for 90 minutes with 10 μM PP1, PP2 or SU6656, followed by agrin treatment overnight in the presence of inhibitors. Inhibitors were added again the next day at the same concentrations for 90 minutes before the start of a precipitation assay.

Precipitation assays and immunoblotting: To examine the association of tyrosine phosphorylation with cortactin or p190RhoGAP, myotubes were rinsed with ice-cold PBS containing 1 mM Na-orthovanadate and 50 mM NaF and extracted at 4ºC in RIPA lysis buffer. The RIPA lysis buffer contained PBS, 1% NP-40, 0.5% Sodium deoxycholate, 0.1% SDS and an excess of protease and phosphatase inhibitors (Wu et al., 2000). Lysates were processed and monoclonal anti-cortactin antibody (clone 4F11), monoclonal anti-RhoGAP (p190) (clone D2D6 from Upstate Biotechnology, Inc. (Lake Placid, NY)) was added for precipitation of cortactin and p190RhoGAP, respectively. As controls, lysis buffer containing only the primary antibody without

cell lysates, or cell lysates without the primary antibody were used. Precipitates were subjected to SDS-PAGE and immunoblotting.

In Western blots, phosphorylation of specific tyrosine residues on cortactin was visualized using polyclonal rabbit antibodies PY421 or PY466 obtained from Biosource International Inc., USA (Camrillo, CA). For detecting overall tyrosine phosphorylation on p190RhoGAP a mixture of monoclonal antibodies PY20 and 4G10 were used. To ensure that the amounts of precipitated proteins were the same in all the lanes a part of the precipitate was loaded and blotted using the precipitating antibody (for example a cortactin IP followed by a cortactin blot). Since the antibody bands migrated differently from the expected protein band such an estimatation was possible. Finally, quantitation of the immunoblots was done by scanning exposed films containing gray, nonsaturated signals with a computerized densitometer (Scantouch 210; Nikon, Tokyo, Japan) and using NIH Image J 1.29X software. The phosphotyrosine signals were divided through the protein signals. Experiments were repeated at least five times to obtain consistent results.

Whole-mount preparation and immunohistochemistry: Soleus muscles were dissected and injected with 2% paraformaldehyde solution for fixation. On PFA fixation the muscle swells, widening the gaps between individual fibres, facilitating isolation of thin fiber bundles. The whole-mount preparations were triple labeled for the following: AChRs were stained using rhodamine-coupled α-bungarotoxin to visualize NMJs. A mixture of rabbit polyclonal antibodies against neurofilament (Sigma, St. Louis, MO) and synaptophysin (Dako, Glostrup, Denmark) followed by Alexa 350 goat anti-rabbit was used to visualize the motor neurons and the nerve endings. The far red channel was used to visualize sub-synaptic cortactin or p190RhoGAP using primary antibodies as described before followed by cyanine 5 (Cy5) conjugated to goat anti-mouse. Conventional microscopy or confocal microscopy with 3D reconstruction (using Imaris 4.1.1) were done as described before in Chapter 2 under Materials and Methods.

Chapter 4

This chapter is adapted from a paper submitted to the EMBO journal. The paper is currently in revision before being sent back to EMBO journal.

My contribution to this paper is in the design and performance of staining experiments and microscopy done with C2C12 and *src*$^{-/-}$;*fyn*$^{-/-}$ myotubes; involving treatments with cholesterol, methyl-β-cyclodextrin and quantitation of AChR clusters. In addition, I advised on biochemical experiments with *src*$^{-/-}$;*fyn*$^{-/-}$ and wild-type myotubes and prepared most of the these cell cultures.

Cholesterol and lipid rafts stabilize the postsynapse at the neuromuscular junction

Running title: postsynaptic stabilization by lipid rafts

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Acknowledgements:
We are grateful to Susanne Erb-Vögtli for cell culture. This work was supported by grants from the Swiss National Science Foundation, the Swiss Foundation for Research on Muscle Diseases, the Zürich Neuroscience Center (to C.F.), and the Novartis Research Foundation (to S.P., A.F.S. and P.C.).

Summary

Stabilization and maturation of synapses are important for development and function of the nervous system. Previous studies have implicated lipid rafts in synapse stabilization, but the underlying mechanisms remain unclear. We found that cholesterol stabilizes clusters of synaptic acetylcholine receptors (AChRs) in denervated muscle in vivo and in nerve-muscle explants in vitro. In paralyzed muscles cholesterol triggered maturation of nerve sprout-induced AChR clusters into pretzel shape. Cholesterol treatment also rescued a specific defect in AChR cluster stability in cultured *src*$^{-/-}$*;fyn*$^{-/-}$ myotubes. Postsynaptic proteins including AChRs, rapsyn, MuSK and Src-family kinases were strongly enriched in lipid rafts prepared from wild-type myotubes. Raft disruption by cholesterol-sequestering methyl-β-cyclodextrin disassembled AChR clusters and decreased AChR-rapsyn interaction and AChR phosphorylation. Amounts of rafts and enrichment of postsynaptic proteins into rafts were decreased in *src*$^{-/-}$*;fyn*$^{-/-}$ myotubes but rescued by cholesterol treatment. These data provide evidence that cholesterol-containing lipid rafts and SFKs act in a dual mechanism in stabilizing the postsynapse: SFKs enhance raft-association of postsynaptic components, whereas rafts provide the microenvironment for SFKs to maintain interactions and phosphorylation of these components.

Introduction

Synaptogenesis is a key process in the development and function of the nervous system. In a first phase, postsynaptic neurotransmitter receptors and associated proteins accumulate underneath active zones of nerve terminals to form a postsynaptic density important in regulating further signaling at the nascent synapse. In cultured neurons, some protein signals triggering postsynaptic differentiation are known, e.g. neurexins and neuroligins (Graf et al., 2004), ephrinB and EphB receptors (Dalva et al., 2000), or Narp (O'Brien et al., 2002). Non-protein factors such as cholesterol are also important: glia-derived cholesterol induces synaptogenesis in cultured retinal ganglion cells (Mauch et al., 2001; Goritz et al., 2005), although its specific role in postsynaptic assembly has not been analyzed. The in vivo relevance of the protein signals and of cholesterol, and many aspects of their mechanism of action remain unknown. In a second phase of the synaptogenesis process, some synapses and postsynaptic densities mature and are stabilized, while others are eliminated. While neural activity is known to regulate this process (Cohen-Cory, 2002), the effector machinery in synapse stabilization is poorly understood.

Cholesterol, along with sphingolipids, is enriched in subcompartments of the cellular membrane system, also known as lipid rafts. These regulate selected intracellular trafficking pathways and signal transduction events through association with signalling proteins (Brown and London, 1998; Simons and Toomre, 2000; Golub et al., 2004). Rafts can act as floating platforms able to diffuse laterally within the plasma membrane, bringing together activated receptors and transducer molecules. Lipid rafts are involved in aspects of synaptic function in cultured cells. Depletion of cholesterol leads to loss of surface AMPA receptors and of synapses in hippocampal neurons (Hering et al., 2003). In ciliary neurons, lipid rafts are necessary for the maintenance of α7 neuronal nicotinic acetylcholine receptors (AChRs) in synapse-associated clusters (Bruses et al., 2001). At the neuromuscular junction (NMJ), the presence of plasmalemmal cholesterol is necessary for proper AChR gating functions (Barrantes, 1993); and AChRs associate with lipid rafts in trafficking toward the plasma membrane in transfected heterologous cells (Marchand et al., 2002). However,

the relevance of rafts and cholesterol for synaptogenesis in vivo, and the identity of the signaling pathways operating through rafts, have remained unclear.

During NMJ formation, myotubes respond to neural agrin, assembling AChRs at nascent synapses (Gautam et al., 1996). This scaffolding function is assigned to MuSK, the trans-membrane kinase that translates agrin into a clustering signal (Glass et al., 1996). Besides MuSK and AChR, rapsyn is the third essential protein for the AChR clustering process (Gautam et al., 1995; Marangi et al., 2001). In response to agrin, the association of rapsyn with AChRs increases and mediates binding to cytoskeletal proteins (Moransard et al., 2003). AChR β subunits become tyrosine-phosphorylated and this modification regulates cytoskeletal linkage and efficient clustering (Borges and Ferns, 2001).

During the maturation of NMJs, plaque-shaped AChR clusters are stabilized and adopt pretzel-shaped configurations, with AChRs located at the crests of postjunctional folds. AChR half-life time is highly increased and synaptic proteins are selectively produced by subsynaptic nuclei (Sanes and Lichtman, 2001). The molecular mechanisms mediating postsynaptic NMJ stabilization differ from those involved in NMJ induction, and much less is known about them (Willmann and Fuhrer, 2002). Essential players include the utrophin-complex with its components dystroglycan and dystrobrevin (Grady et al., 2000; Jacobson et al., 2001), and Src-family kinases (SFKs). These kinases are activated by agrin (Mittaud et al., 2001) and maintain AChR-rapsyn interaction and AChR β phosphorylation (Sadasivam et al., 2005). In cultured *src*$^{-/-}$*;fyn*$^{-/-}$ myotubes, agrin- or laminin-induced AChR clusters are unstable and disassemble rapidly after withdrawal of these factors (Smith et al., 2001; Marangi et al., 2002). Interfering with SFK function in vivo causes postsynaptic disintegration of adult NMJs (Sadasivam et al., 2005). Since SFK functions are specifically associated with lipid rafts in other cells (Resh, 1999; Simons and Toomre, 2000), these results have raised the possibility that raft-dependent processes might be involved in postsynaptic apparatus maintenance through SFKs.

To investigate mechanisms of postsynaptic maturation, we determined whether, and through what signaling molecules, cholesterol and lipid rafts might stabilize NMJs in vivo and in vitro. We find that cholesterol addition stabilizes NMJs and

promotes their maturation from patch- to pretzel-type configurations. Postsynaptic proteins reside in cholesterol-rich lipid rafts, and raft dispersion disrupts AChR clusters, AChR-rapsyn interaction and AChR β phosphorylation. In *src*$^{-/-}$;*fyn*$^{-/-}$ myotubes, cholesterol addition normalizes the reduced raft assocation of postsynaptic proteins and stabilizes AChR clusters. These results suggest a dual mechanism for postsynaptic cluster stabilization through Src-family kinases, involving an enhancement of the association of cluster components with cholesterol-containing lipid rafts, and interactions and phosphorylation of these components at rafts.

Results

Cholesterol stabilizes AChR clusters in vivo

To investigate a possible role of cholesterol and lipid rafts in promoting postsynaptic apparatus maintenance in vivo, we analyzed the state of assembly of AChR clusters at denervated NMJs in the absence or presence of exogenous cholesterol. The sciatic nerve was cut in 1-month old mice, and AChR clusters were visualized 12 days later in two DeSyn muscles (lateral gastrocnemius and medial gastrocnemius), which exhibit substantial postsynaptic cluster disassembly under these experimental conditions (Pun et al., 2002). To visualize denervated synaptic sites we counterstained muscle sections with an antibody against p75, a protein upregulated in Schwann cells in the absence of nerve contact (Taniuchi et al., 1986). As expected, denervated synaptic sites exhibited only remnants of AChR clusters after 12 d of denervation (Fig. 1A; note irregular AChR labeling patterns, with only small regions of intense labeling). In contrast, when cholesterol was applied daily to denervated muscles, starting 5 days after denervation, AChR signals at denervated synaptic sites were much better preserved (Fig. 1A; note that p75 signals were not affected by the cholesterol treatment). These AChR signals were comparable to clusters in non-denervated control animals (not shown, but refer to an earlier paper (Pun et al., 2002)). A quantitative analysis of AChR labeling intensities revealed that synaptic sites had lost most of their AChR signal 12 d after denervation, but that the synaptic signal was largely preserved in the presence of exogenous cholesterol (Fig. 1C).

To investigate AChR cluster protection by cholesterol under more challenging experimental conditions, we analyzed nerve-muscle explant preparations of soleus maintained at 37°C in Ringer solution supplemented with calcium. To reliably identify synaptic sites we carried out these experiments using transgenic mice expressing a synaptophysin-GFP construct in neurons (*Thy1-spGFP^{mu}*) (De Paola et al., 2003). Under these experimental conditions, many synaptic sites lost most of their AChR signal after 3 h ex vivo, such that about half the synapses appeared normal while others had only low-intensity AChR label (Fig. 1B, C). Inclusion of the cholesterol sequestering agent methyl-β-cyclodextrin, which disrupts lipid rafts (Simons and Toomre, 2000; Tansey et al., 2000; Ma et al., 2003), accelerated the loss of AChR signal (Fig. 1B, C). In contrast, inclusion of cholesterol in the culture medium protected most AChR clusters (Fig. 1B, C).

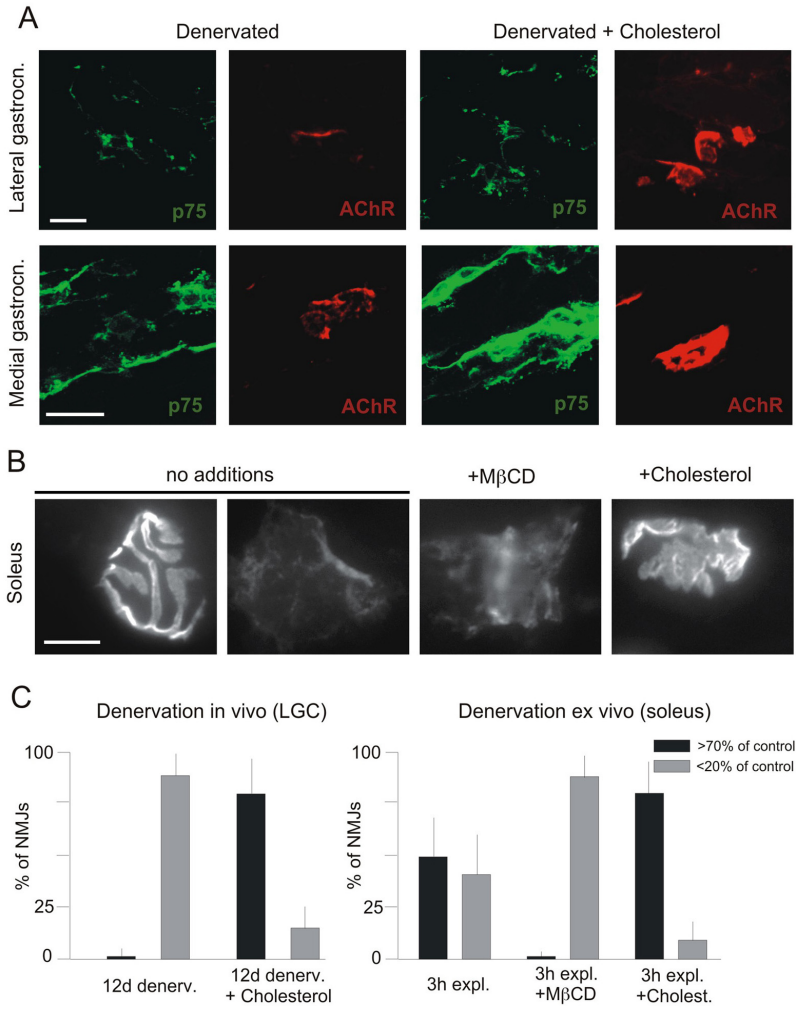


Fig. 1. Cholesterol stabilizes AChR clusters in denervated muscles. (A) Appearance of AChR clusters in two DeSyn muscles 12 days after denervation. Sciatic nerves were cut in 1-month mice; the absence of intact axons is confirmed by the expression of p75 in Schwann cells. Where indicated, cholesterol was applied daily, starting 5 days after denervation. (B) Examples of AChR clusters (visualized by rhodamine-α-bungarotoxin; RITC-α-BT) in soleus nerve-muscle explants after 3 hours in vitro. (C) Quantitative analysis of data as shown in (A) (left) and (B) (right). AChR labeling intensities (RITC-α-BT) were compared to controls; shown are fractions of NMJs with signal at least 70%, or less than 20% of control values. N=300 AChR clusters (from 3 mice each). Bars: 40 (A) and 20 μm (B).

To determine whether cholesterol might also promote the assembly of new AChR clusters in vivo, we used reporter mice expressing membrane-targeted GFP in neurons (*Thy1-mGFP*^{s}) (De Paola et al., 2003) and carried out cholesterol supplementation experiments in lateral gastrocnemius muscle chronically treated with Botulinum toxin A. These experimental conditions (lateral gastrocnemius in 1-month old mice; toxin applications every 4th day for a total of 20 days) induce the disassembly of postsynaptic apparatus at NMJs, a massive nerve sprouting response, and induction of small ectopic AChR plaques along the nerve sprouts (Fig. 2A, B (left panels); see also (Santos and Caroni, 2003). Daily local applications of cholesterol from day 10 of the BotA treatment, i.e. at a time when NMJ disassembly and nerve sprouting were not yet pronounced (Santos and Caroni, 2003), led to a suppression of the AChR cluster disassembly process, which was accompanied by a suppression of nerve sprouting and of ectopic AChR plaque induction by sprouts in these paralyzed muscles (Fig. 2A (right panels)). The resulting AChR signals appeared very similar to those in non-treated control animals (not shown; but refer to (Santos and Caroni, 2003). Significantly, initiation of the cholesterol treatment at day 15, when sprouting was well advanced (Santos and Caroni, 2003), led to the assembly of large, pretzel-shaped ectopic AChR clusters along the sprouts (Fig. 2B).

Taken together, these data provide evidence that local applications of exogenous cholesterol in vivo protect AChR clusters against denervation-induced disassembly, and promote the maturation of sprout-induced ectopic AChR clusters in paralyzed muscles from an embryonic-type plaque shape into a pretzel shape. We thus propose

that cholesterol is an important factor for the maturation and stabilization of the NMJ in vivo.

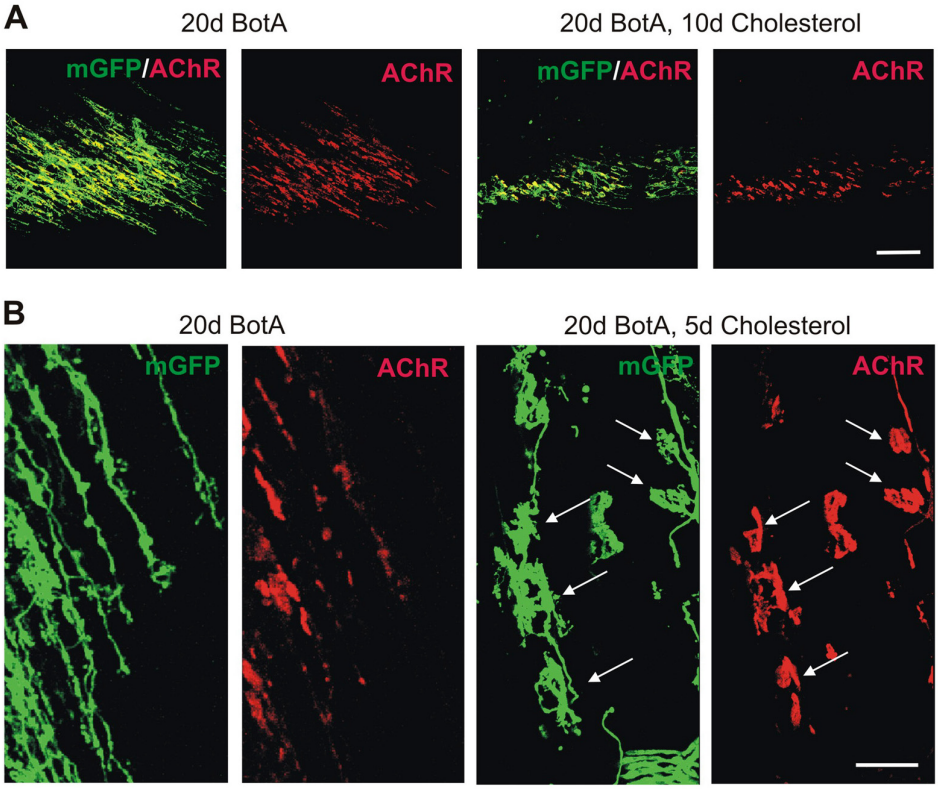


Fig. 2. Cholesterol promotes AChR cluster assembly at original and ectopic NMJs in paralyzed DeSyn muscles. Low- (A) and high-magnification (B) views of presynaptic nerves (mGFP) and postsynaptic AChR clusters (RITC-α-BT) in lateral gastrocnemius muscles treated with Botulinum toxin A (BotA). The chronic BotA treatment elicited a massive nerve sprouting response in this DeSyn muscle; cholesterol promoted AChR cluster assembly, and inhibited nerve sprouting. Note pretzel-shaped AChR clusters (arrows, right) induced by sprouts (arrows left) in the presence of exogenous cholesterol. Bars: 200 (A) and 40 μm (B).

Cholesterol stabilizes AChR clusters in cultured src$^{-/-}$;fyn$^{-/-}$ myotubes

To analyze the mechanism of action of cholesterol in stabilizing AChR clusters, we turned to aggregation assays in cultured myotubes. Furthermore, we took advantage of cells from mice lacking Src and Fyn, where AChR clusters are normally induced by agrin or laminin treatment, but disassemble within a few hours after removal of these factors from the medium (Smith et al., 2001; Marangi et al., 2002). We treated *src*$^{-/-}$;*fyn*$^{-/-}$ myotubes with agrin to induce maximal AChR clustering, then withdrew

agrin and determined whether the addition of cholesterol might stabilize AChR clusters. We found that after 5 h, cholesterol-treated cells showed the same number of AChR clusters as cells from which agrin was not withdrawn (Fig. 3). Cells from which agrin was withdrawn for 5 h, without addition of cholesterol, showed a low cluster number, comparable to the level of spontaneous clustering. In wild-type cells, clusters were very stable following removal of agrin, as published previously (Smith et al., 2001; Marangi et al., 2002). This stability prohibited assessing significant effects of cholesterol.

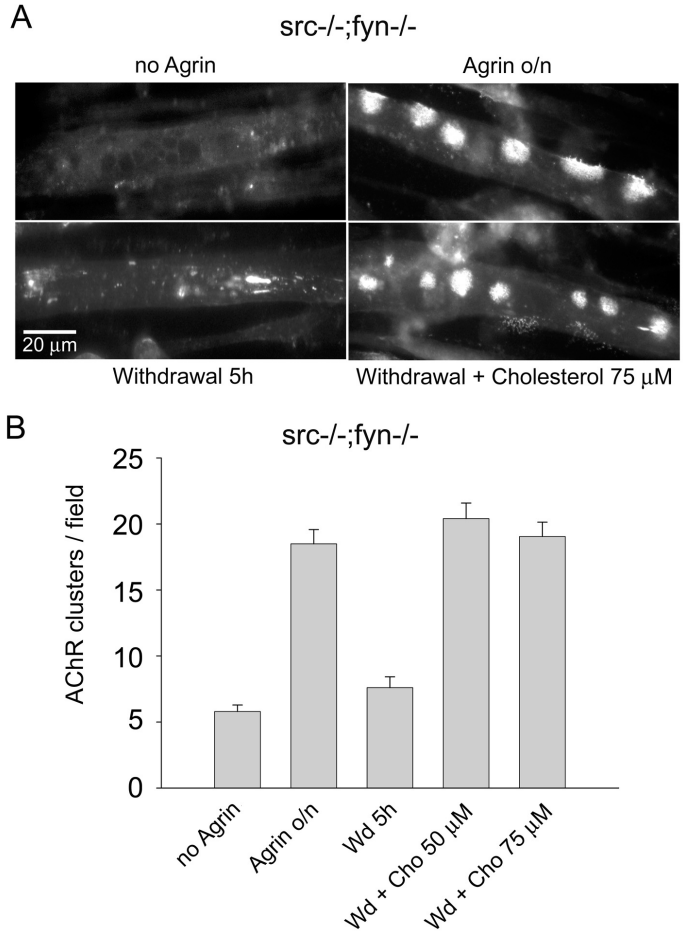


Fig. 3. Cholesterol stabilizes AChR clusters in *src*$^{-/-}$;*fyn*$^{-/-}$ myotubes. (A) *src*$^{-/-}$;*fyn*$^{-/-}$ myotubes were not treated or stimulated overnight with 1 nM agrin to induce AChR clusters (top row). Agrin was withdrawn, cells were washed and incubated for 5 h in agrin-free medium lacking (bottom left) or containing (bottom right) 75 µM cholesterol. Myotubes were stained with rhodamine-α-BT to visualize AChR clusters. (B) For cluster quantification, visual fields covering about 3 times the area of a panel shown in A were taken and only compact clusters with a mimimum size of 5 µm were counted.

We next determined whether cholesterol might compensate for agrin withdrawal by enhancing signaling processes involved in the formation of the NMJ. Cholesterol addition to wild-type myotubes did not induce formation of AChR clusters (Fig. 4A). In addition, cholesterol did not lead to phosphorylation of MuSK or the β subunit of AChRs, unlike agrin (Fig. 4B, C). AChR β phosphorylation is a known requirement of efficient receptor clustering and cytoskeletal linkage (Borges and Ferns, 2001). Taken together, our data show that cholesterol stabilizes AChR clusters in cultured myotubes but does not activate agrin/MuSK signaling.

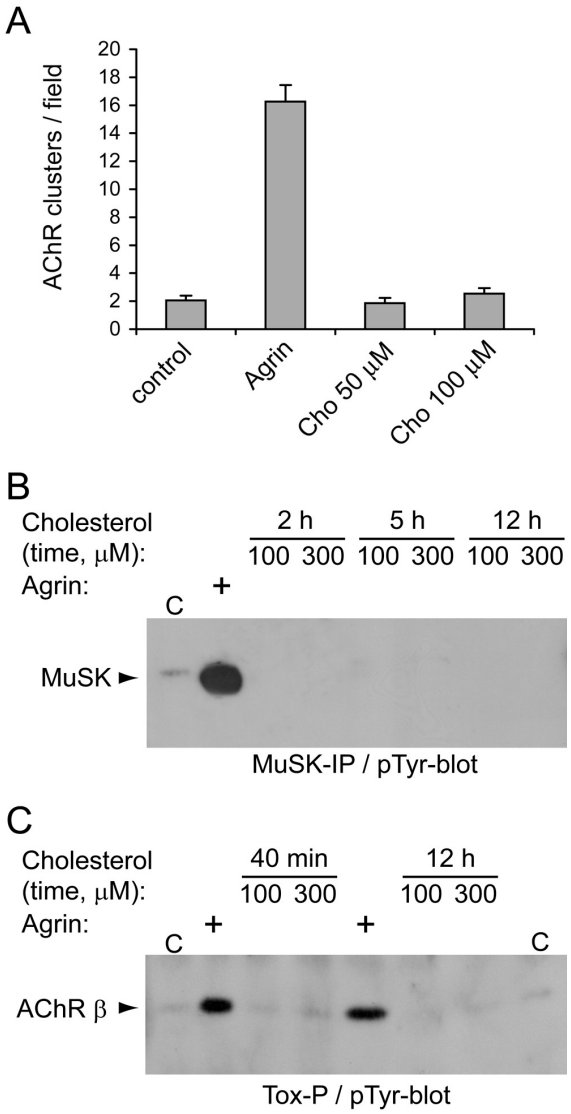


Fig. 4. Cholesterol does not induce AChR clustering and phosphorylation of MuSK and AChR β subunits. (A) Cholesterol or 1 nM agrin were added overnight to C2C12 myotubes. Cells were stained with rhodamine-α-BT and AChR clusters quantitated as in Figure 3. (B and C) C2C12 myotubes were

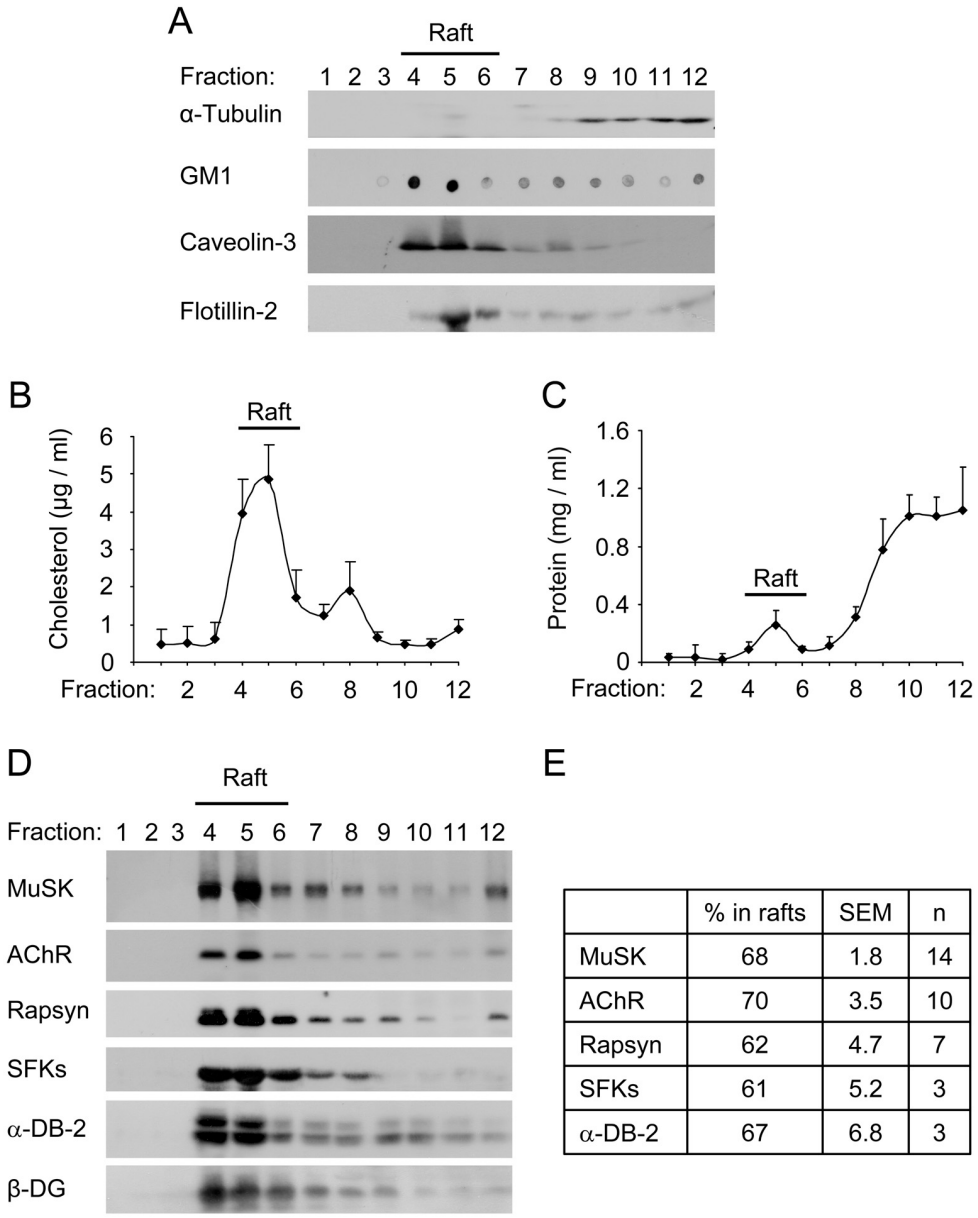
treated with different doses of cholesterol, or with 1 nM agrin for 40 min, as indicated; c, untreated control. From cell lysates, MuSK was immunoprecipitated (B) or AChRs were precipitated using biotin-α-BT and streptavidin-agarose (Tox-P, C). Phosphotyrosine immunoblotting detected phosphorylation of MuSK and AChR β subunits. The identity of these phosphoproteins was confirmed by reprobing with MuSK- or AChR β-specific antibodies (not shown).

The proteins involved in AChR cluster stabilization reside in cholesterol-rich lipid rafts in muscle

Cholesterol is a key component of lipid rafts, and its action in cluster stabilization might involve raft-dependent processes. We therefore prepared and analyzed rafts from cultured wild-type myotubes using a well-established protocol (Song et al., 1996; Song et al., 1996; Riddell et al., 2001; Nishio et al., 2004; Rhainds et al., 2004; Zhang et al., 2005). Cell homogenates were floated on discontinuous sucrose gradients, and fractions were analyzed by immunoblots. The raft fractions (4-6) were found at the interface between 5% and 35% sucrose and defined by strong enrichment of typical raft markers such as caveolin-3, flotillin-2, cholesterol and the sphingolipid, ganglioside GM1 (Fig. 5A, B). Measurement of protein concentration showed that fractions 4-6 contain little of the overall protein (only 9.5 ± 0.2%; mean ± SEM, n=5); most protein was found at the bottom of the gradient, in fractions 8-12 which contain free (non-raft associated) cellular proteins (Fig. 5C). Another negative control was α-tubulin, which did not associate with rafts and was mostly recovered in the free fractions (Fig. 5A). These controls established the validity of the method for preparation of cholesterol-enriched lipid rafts from myotubes.

We probed fractions for the content of postsynaptic proteins. We found the AChR highly enriched in rafts, as 70% of the total receptor present in all fractions of the gradient resided in fractions 4-6 (Fig. 5D, E). This reflects a 7.3-fold enrichment when compared to the profile of bulk protein (Fig. 5C). Like AChRs, elements of the agrin signaling pathway such as MuSK and rapsyn were similarly concentrated in rafts. The same was true for SFKs (Fig. 5D, E), which are known from other cell types to be typical constituents of lipid rafts (Resh, 1999; Simons and Toomre, 2000). Finally, β-dystroglycan and α-dystrobrevin-2, members of the utrophin-complex important for NMJ stabilization (Grady et al., 2000; Jacobson et al., 2001), were also

recovered efficiently in rafts. An overnight incubation with agrin, sufficient to produce maximal AChR clustering, did not detectably affect the fractions of AChR, rapsyn and MuSK partitioning into lipid rafts (data not shown).



	% in rafts	SEM	n
MuSK	68	1.8	14
AChR	70	3.5	10
Rapsyn	62	4.7	7
SFKs	61	5.2	3
α-DB-2	67	6.8	3

Fig. 5. Postsynaptic proteins associate with cholesterol-rich lipid rafts in myotubes. Rafts were prepared from wild-type myotubes (C2C12 or clones SW10 and SW5), and fractions of the discontinuous sucrose gradients were collected. Fractions 9-12 represent the bottom gradient step (45% sucrose) containing the total cell extract. Fractions 5-8 represent the 35% sucrose layer and fractions 1-4 the top layer (5% sucrose). (A) Fractions were analyzed by immunoblotting (α-tubulin, caveolin-3, flotillin-2) or dot blotting (ganglioside GM1). Fractions 4-6 contain lipid rafts and α-tubulin served as a negative control. (B) Fractions were analyzed for the content of cholesterol, showing high enrichment

in raft fractions 4-6 (n=4). (C) Protein assays of gradient fractions reveal the bulk of protein in the bottom fractions, illustrating the specificity of the raft preparation (n=4). (D) Fractions were subjected to immunoblotting, showing that MuSK, AChRs (β-subunit), rapsyn, SFKs, α-dystrobrevin-2 (α-DB-2) and β-dystroglycan (β-DG) all partition efficiently into rafts. (E) Blots as shown in D were quantitated by densitometric scanning. For each protein, the intensities of bands in raft fractions 4-6 were related to the sum of all fractions to quantify the percentage in rafts (n=number of experiments).

Dispersion of lipid rafts disrupts AChR clusters, AChR-rapsyn interaction and AChR β phosphorylation

Besides raft-association of proteins, another standard tool to investigate the role of lipid rafts in a given cellular process is to disrupt the rafts by methyl-β-cyclodextrin (MβCD). We treated wild-type myotubes overnight with agrin to induce maximal AChR clusters and then added MβCD for 1-1.5 hrs. The number of clusters of normal size and morphology was strongly reduced by MβCD (Fig. 6). Upon MβCD treatment, we noticed many smaller clusters and areas containing cluster fragments. The myotube morphology was unaffected, and following removal of MβCD the myotubes lived for extended periods of time and formed normal agrin-induced stable AChR clusters, like untreated controls (data not shown). This indicates that the MβCD effect was specific and not a consequence of impaired myotube health. These data thus show that the integrity of cholesterol-containing rafts is required to maintain the accumulation of large focal AChR clusters at the cell surface.

Maintenance of the AChR-rapsyn interaction and of AChR β phosphorylation depends on SFKs and is crucial in maintaining clusters (Sadasivam et al., 2005). We therefore analyzed the role of lipid rafts in these processes. Myotubes were again treated overnight with agrin to induce maximal clustering, followed by addition of 5 mM MβCD for 1.5 h. AChRs were isolated from cell lysates, and associated rapsyn or phosphotyrosine content determined by immunoblotting. We found that the agrin-induced increase in AChR-rapsyn interaction was disrupted by MβCD (Fig. 6C). Likewise, agrin-induced phosphorylation of AChR β was reduced to basal levels by MβCD (Fig. 6D).

Taken together, these results show that dispersion of lipid rafts prevents the maintenance of large focal AChR clusters by disrupting agrin-induced AChR-rapsyn interaction and AChR phosphorylation.

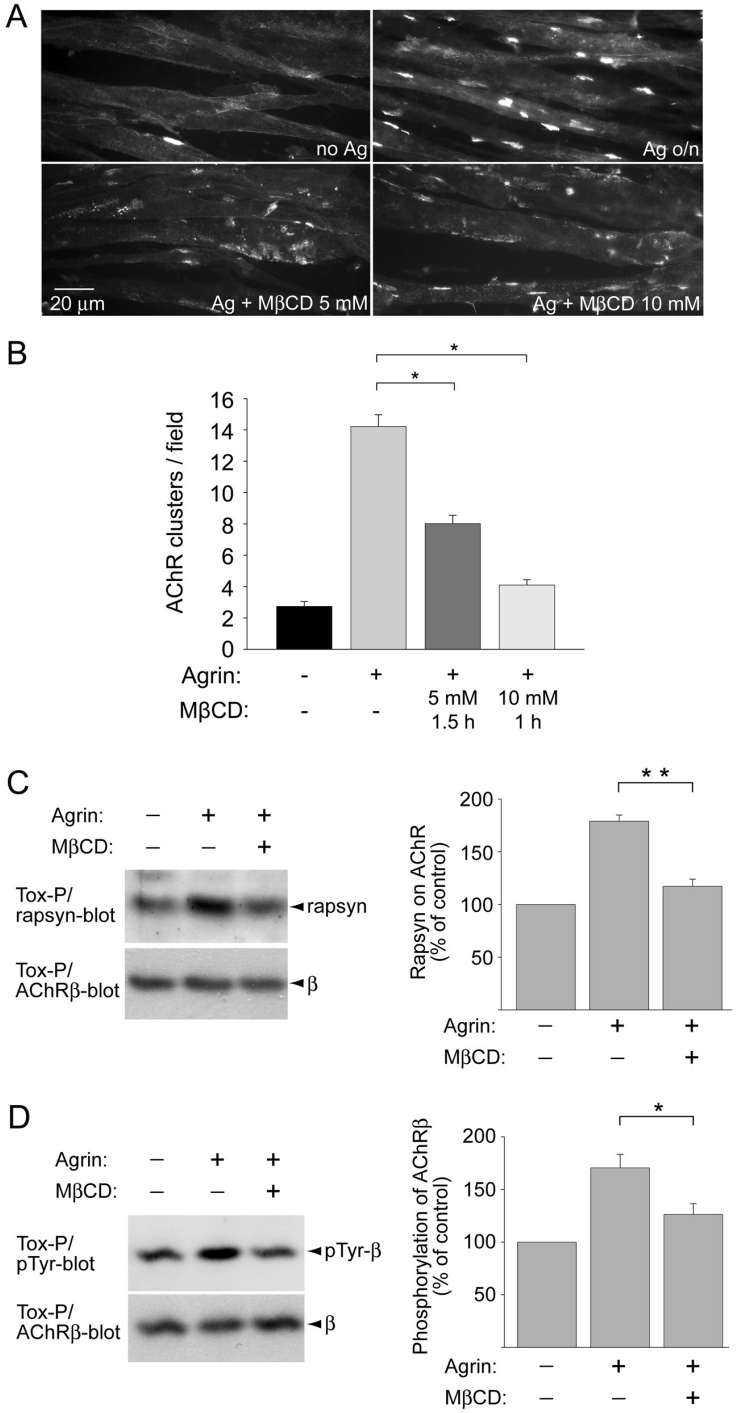


Fig. 6. MβCD disrupts AChR clusters, AChR-rapsyn interaction and AChR β phosphorylation in C2C12 myotubes. (A) C2C12 myotubes where first treated overnight with agrin (Ag) to induce AChR clusters. MβCD was then added in the continued presence of agrin, causing AChR clusters to fragment

and disappear, as revealed using rhodamine-α-BT staining. (B) Clusters of 5 μM minimal size were quantitated. (C, D) Cells were treated with agrin and MbCD (5 mM, 1.5 h) as in A. AChRs were precipitated from cell lysates using biotin-α-BT (Tox-P). In immunoblots, AChR-associated rapsyn (C), phosphorylation of AChR β (D) and AChR β itself (C, D) were detected and quantified by densitometric scanning (C, n=4; D, n=8).

Impaired partitioning of postsynaptic proteins into rafts in the absence of Src and Fyn, and rescue by cholesterol

To further define the molecular mechanism through which cholesterol and rafts stabilize AChR clusters, we analyzed the composition of rafts prepared from *src*$^{-/-}$*;fyn*$^{-/-}$ myotubes, in which clusters are unstable. Like those from wild-type cells, rafts from mutant myotubes were enriched for ganglioside GM1, caveolin-3 and flotillin-2 (Fig. 7A). A cholesterol profile revealed enrichment in rafts, but to a lesser extent than in wild-type cells (Fig. 7B). Rafts from *src*$^{-/-}$*;fyn*$^{-/-}$ myotubes contained little overall protein (Fig. 7C), as did wild-type rafts. Interestingly, significantly less of the total AChR and MuSK were in rafts from *src*$^{-/-}$*;fyn*$^{-/-}$ myotubes when compared to wild-type, the decrease being 30% for AChRs and 23% for MuSK (Fig. 7D).

The overproportionally decreased raft association of AChRs in *src*$^{-/-}$*;fyn*$^{-/-}$ myotubes could have two reasons: Src and Fyn may maintain normal numbers of rafts in a myotube and/or act as a recruitment signal that brings postsynaptic proteins (such as AChRs) into rafts. To investigate these possibilities, we quantitated raft partitioning of typical raft markers. 19% less of total cholesterol were found in the raft fractions 4-6 in *src*$^{-/-}$*;fyn*$^{-/-}$ myotubes when compared to wild-type (Fig. 7E) and the trend was similar for caveolin-3 (Fig. 7F). Overall cellular levels of cholesterol, quantified per μg of cellular protein, were normal in the mutants, excluding overall nonspecific effects from the lack of Src and Fyn (Fig. 7G). These data suggest that *src*$^{-/-}$*;fyn*$^{-/-}$ myotubes have less rafts than wild-type cells. The reduction in rafts however appears smaller than the reduction in raft-association of AChRs. Thus, Src and Fyn most likely also act as a recruitment factor for AChRs (and MuSK) into rafts.

Importantly, cholesterol addition to *src*$^{-/-}$*;fyn*$^{-/-}$ myotubes not only stabilized AChR clusters (Fig. 3), but restored the raft partitioning of AChRs and MuSK back to

normal (Fig. 7D). Likewise, the raft enrichment of cholesterol itself and of caveolin were normalized by cholesterol treatment (Fig. 7E, F). Thus, while the absence of Src and Fyn decreases the number of rafts and the recruitment of postsynaptic proteins into rafts, cholesterol addition overcomes this, normalizing the enrichment of AChR and MuSK in rafts. Taken together, these loss- and gain-of-function data thus strongly implicate a role of intact rafts, through SFKs, in AChR cluster stabilization.

Figure 7

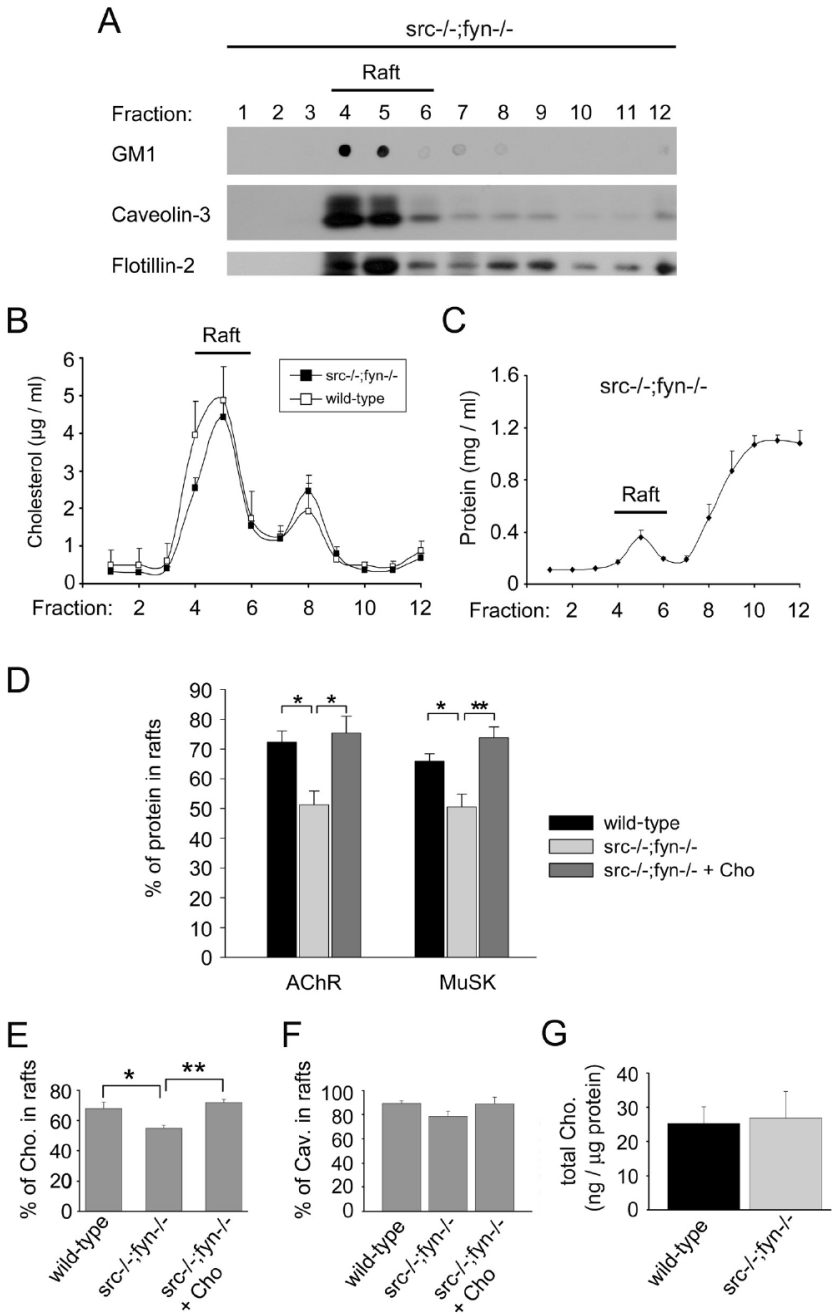


Fig. 7. In *src*$^{-/-}$;*fyn*$^{-/-}$ myotubes, raft association of postsynaptic proteins is reduced but restored by cholesterol. (A-C) Characterization of rafts in *src*$^{-/-}$;*fyn*$^{-/-}$ myotubes. Rafts were prepared from clones DM11 or DM15, and the content, in gradient fractions, of ganglioside GM1, caveolin-3, flottilin-2 (A), cholesterol (B, n=12) and total protein (C, n=4) was analyzed. Markers are concentrated in raft fractions 4-6, with overall protein enriched at the gradient bottom (negative control). Cholesterol is less enriched in rafts than in wild-type cells (B; we show the profile from Fig. 5B for comparison) (D) Gradient fractions were analyzed for the content of AChR, rapsyn and MuSK, and the percentage of these proteins in raft fractions 4-6 was quantified as in Figure 5E. Wild-type cells (C2C12 or clones SW5 and SW10; n=3-7), *src*$^{-/-}$;*fyn*$^{-/-}$ myotubes (n=4-6) and *src*$^{-/-}$;*fyn*$^{-/-}$ myotubes treated with cholesterol (n=4-5) were used. *src*$^{-/-}$;*fyn*$^{-/-}$ myotubes have significantly lower percentages of proteins in rafts, and cholesterol restores this in the case of AChR and MuSK. (E) Cells were treated as in D and the percentage of cholesterol in raft fractions 4-6 was quantitated. Raft association of cholesterol is lower in *src*$^{-/-}$;*fyn*$^{-/-}$ myotubes (n=12) than in wild-type myotubes (n=8). Addition of cholesterol to the cell culture medium restores the amount of cholesterol in the raft fractions to the levels of wild-type myotubes (n=6). (F) Analysis as in E, examining caveolin-3 (Cav.). (G) The total amount of cholesterol, detected in total cell extracts, is the same in wild-type and *src*$^{-/-}$;*fyn*$^{-/-}$ myotubes (n=8).

Discussion

We have shown that cholesterol is an important factor for the stabilization and maturation of the NMJ in vivo and in vitro. This involvement of cholesterol does not stem from activation of the agrin/MuSK signaling pathway, but from promoting the incorporation of postsynaptic proteins into lipid rafts. We provide evidence that lipid raft integrity is important for the maintenance of AChR clusters, and that SFKs trigger postsynapse stabilization by enhancing the association of critical postsynaptic proteins with lipid rafts. Rafts, in turn, allow SFKs to phosphorylate the AChR and to maintain AChR-rapsyn interaction. These data show that concerted action of cholesterol with lipid rafts and SFKs forms a mechanism for stabilization of the postsynapse of NMJs.

Cholesterol promotes postsynaptic stabilization in vivo and in cultured myotubes

We provide the first evidence that cholesterol promotes synaptic stability in vivo. Cholesterol addition to denervated adult DeSyn gastrocnemius muscle during 12 days prevented the postsynaptic disassembly that would normally occur. Furthermore,

soleus nerve-muscle explants exhibited substantial AChR pretzel disassembly within 3 hours, which was largely prevented by cholesterol treatment. Finally, Botulinum toxin A caused massive nerve sprouting and induction of plaque-shaped AChR clusters along the sprouts. When applied before this response, cholesterol stabilized existing AChR pretzels at NMJs; when applied after massive sprouting had started, cholesterol treatment promoted maturation of sprout-induced AChR cluster to adopt adult-type pretzel shape. Cholesterol addition, at the time of agrin withdrawal, also stabilized AChR clusters in cultured *src*$^{-/-}$;*fyn*$^{-/-}$ myotubes.

Conversely, sequestering cholesterol by MβCD treatment, leading to disruption of lipid rafts, accelerated the disassembly of AChR pretzels in explants of soleus muscle, and it disrupted clusters of AChRs in wild-type myotubes in culture. These data establish cholesterol as a factor for the stabilization and maturation of the postsynaptic apparatus at the NMJ.

Interestingly, the mechanism of cholesterol action in postsynaptic stabilization does not involve a re-activation of those pathways that lead to the formation of the NMJ: we find that cholesterol addition to myotubes does not cause AChR clustering, activation of MuSK or phosphorylation of AChR β subunits. Since cholesterol nonetheless stabilizes AChR clusters, this shows that the pathways for stabilization of postsynaptic clusters are different from those for induction of cluster formation.

Cholesterol action in postsynaptic stabilization occurs via lipid rafts

To investigate the mechanism of action of cholesterol, we used myotubes in culture. We found that AChRs, rapsyn, MuSK, SFKs, α-dystrobrevin-2 and β-dystroglycan were highly concentrated in rafts, and the degree of concentration, ca. 70% of total, was similar to cholesterol. Importantly, cholesterol addition to cultured *src*$^{-/-}$;*fyn*$^{-/-}$ myotubes increased the raft association of AChRs and MuSK. It also augmented raft participation of cholesterol in these cells (see Fig. 7E), suggesting that the cholesterol treatment increased the number of lipid rafts.

These observations, together with the stabilizing effect of cholesterol on AChR clusters and the disassembly of clusters by the raft-disrupting agent MβCD, lead to the conclusion that cholesterol and lipid rafts are key players in stabilizing the

postsynaptic apparatus of NMJs, by promoting the raft-association of postsynaptic proteins.

Cholesterol, lipid rafts and SFKs: a core mechanism for stabilization of the postsynapse

We used *src*$^{-/-}$;*fyn*$^{-/-}$ myotubes to investigate signaling pathways by which cholesterol and lipid rafts stabilize clusters of AChRs. The following observations indicate that cholesterol and rafts act in concert with SFKs in postsynaptic stabilization. First, the absence of Src and Fyn (Smith et al., 2001; Marangi et al., 2002), like the disruption of lipid rafts by MβCD (Fig. 6), cause disassembly of AChR clusters in cultured myotubes. In vivo, SFKs maintain adult NMJs (Sadasivam et al., 2005), similar to the stabilizing action of cholesterol (Fig. 1, 2). Second, SFKs themselves are enriched in lipid rafts, suggesting that their action in postsynaptic stabilization may occur through the rafts. In cultured *src*$^{-/-}$;*fyn*$^{-/-}$ myotubes, cytoskeletal linkage of AChRs is weakened and, following agrin withdrawal, AChR β phosphorylation and AChR-rapsyn interaction rapidly decrease (Sadasivam et al., 2005). We show here that addition of MβCD to agrin-treated wild-type myotubes produces the same effect, as it reduces AChR-rapsyn interaction and AChR β phosphorylation. Thus, lipid rafts allow SFKs to act in the stabilization of AChR clusters by phosphorylating the AChR and maintaining its link with rapsyn and the cytoskeleton. Third, in *src*$^{-/-}$;*fyn*$^{-/-}$ myotubes raft association of postsynaptic proteins is reduced due to both a decrease in the raft number and a loss of Src-Fyn-dependent recruitment of postsynaptic proteins into the rafts. Cholesterol addition to *src*$^{-/-}$;*fyn*$^{-/-}$ myotubes restores raft numbers, raft association of postsynaptic proteins and stability of AChR clusters.

Based on these results we propose a mechanism involving a reciprocal relationship between Src, Fyn and rafts in AChR cluster stabilization. In the first aspect of this dual mechanism, Src and Fyn mediate raft integrity and recruitment of postsynaptic apparatus components into rafts. Raft integrity is known to involve an optimal balance between raft-lipids and raft-proteins: upon addition of excess ganglioside GM1 to cultured MDCK cells, raft-proteins participate to a lesser degree into rafts, since the

lipid-protein balance in rafts is disturbed (Simons et al., 1999). In a similar fashion, absence of the prominent raft components Src and Fyn, which contain both lipid (double acylation) and protein parts, may cause deranged lipid-protein ratios in rafts, leading to a decrease in functional rafts. Treatment with cholesterol overcomes this, most likely because exogenous cholesterol is incorporated into the plasma membrane and restores lipid-protein ratios in rafts independently of Src and Fyn.

Recruitment of proteins into rafts is triggered by lipid modifications that act as anchors, a prominent example being GPI-linked proteins. SFKs are targeted to rafts through their fatty acyl groups (Resh, 1999). AChRs and MuSK lack such modifications but interact with SFKs (Fuhrer and Hall, 1996; Mohamed et al., 2001); and AChRs associate with several postsynaptic proteins including utrophin (Fuhrer et al., 1999), which is linked to F-actin (Winder et al., 1995). These protein interactions are likely to form a basis for our observed SFK-dependent recruitment of postsynaptic proteins into rafts, and the interactions contribute to raft stabilization through link to the actin cytoskeleton.

In the second aspect of the dual mechanism for AChR cluster stabilization, rafts in turn create the required lipid and protein microenvironment for Src and Fyn to act in the NMJ stabilization pathway. Tyrosine kinases in muscle are known to be counteracted by phosphatases that control, for example, the end level of AChR and MuSK phosphorylation (Wallace, 1994; Madhavan et al., 2005). Incorporation of postsynaptic components in lipid rafts may protect them from phosphatases, allowing kinases of the Src-family to act in NMJ stabilization. This action involves enhancement of protein modifications and of key protein interactions, such as phosphorylation of the AChR β subunit and interaction of AChR with rapsyn.

Besides AChR and rapsyn, SFKs are likely to have other downstream target molecules in stabilizing the postsynapse, most likely cytoskeletal organizers (Sadasivam et al., 2005). Lipid rafts may participate in the regulation of such downstream pathways. In agreement with such a proposal, both SFKs and lipid rafts are known in other cell types to promote signalling interactions that locally organize cytoskeletal elements, e.g. by favoring activation of Rho-like GTPases and promoting actin assembly (Golub et al., 2004; Rodgers et al., 2005), or by organizing

microtubules (Cox and Maness, 1993; del Pozo et al., 2004; Palazzo et al., 2004). Thereby specialized domains can be formed at the cell surface as represented by focal adhesion sites, and these specializations are stabilized through many participating cytoskeletal elements.

In summary, our work shows that cholesterol-rich lipid rafts represent a microenvironment for postsynaptic NMJ proteins. The rafts are formed due to SFK action and in turn allow these kinases to promote key phosphorylations and protein interactions for maintenance of the postsynaptic apparatus. The downstream substrates in this cascade remain to be investigated.

Materials and methods

In vivo and ex vivo experiments. *Thy1-mGFP*^{s} and *Thy1-spGFP*^{mu} reporter mice were as described elsewhere (De Paola et al., 2003); all treatments were initiated when mice were 1-month old. Drugs were injected locally, subcutaneously (100 µl/mouse injection volumes). Botulinum toxin A (Allergan AG, Lachen, Switzerland) was applied at 0.02 U/g body weight every 4th day. Where indicated, cholesterol was applied daily (50 µM in injection solution). Nerve-muscle explants of soleus were maintained at 37°C in calcium-supplemented Ringer solution for 3 hours, and then labeled with RITC-α-BT (2 µg/ml) for the analysis of AChR clusters. Where indicated, drugs in the incubation medium were 50 µM (cholesterol) and 10 mM (MβCD). Cryostat sections of unfixed muscles were postfixed (10 min, 3.5% formaldehyde in PBS) and labeled for immunocytochemistry as described before (Pun et al., 2002). Fluorescent data were imaged and acquired using an Olympus (BX61) confocal microscope, Fluoview 4.1 software, and identical settings for all experiments belonging to one set (denervation, nerve-muscle explants, paralysis experiments). NMJ labeling intensities (integral of RITC-α-BT signal at individual NMJs) were derived from z-stacks using ImageJ software. Only NMJs lying en-face with respect to the plane of imaging were included in the analysis. For paralysis experiments, we analyzed muscle innervation patterns using reporter mice expressing mGFP in neurons (*Thy1-mGFP*^{s}) and, whole mount preparations of muscles. Briefly, identified

muscles were dissected, fixed in PBS with 3.5% formaldehyde (30 min, room temperature), washed, and counterstained with RITC-α-BT (2 μg/ml).

Cell cultures and treatments. C2C12, *src*$^{-/-}$*;fyn*$^{-/-}$ (clones DM11 and DM15), and their corresponding wild-type cells (clones SW5 and SW10) were propagated and fused to form myotubes as described earlier (Smith et al., 2001; Marangi et al., 2002). To induce maximal AChR cluster formation, cells were treated with 1 nM recombinant neural agrin C-Ag$_{12,4,8}$ (Fuhrer et al., 1997) overnight for 16 h. To withdraw agrin, cells were washed and incubated in agrin-free medium; this procedure was used before and shown to be efficient in removing the vast majority of agrin from cells (Mittaud et al., 2004). For cholesterol treatment, water-soluble cholesterol (Sigma; Fluka, Switzerland) was aliquoted in PBS, diluted in fusion medium immediately prior to use, and used at a final concentration of 50 μM if not specified otherwise. In methl-β-cyclodextrin (MβCD) treatments, MβCD (Sigma) was diluted in fusion medium at 100 mM and used at a final concentration of 10 mM or 5 mM. We confirmed effective depletion of cholesterol from the cell membrane after incubation with 5 mM MβCD for 40-60 min: total cellular cholesterol content was reduced to 50-65% of untreated controls, and the use of fetal bovine serum-containing medium during the treatment had no effect on the cholesterol depletion (data not shown).

C2C12, SW5 and SW10 cells gave identical results in all assays and we refer to them commonly as wild-type cells. Likewise, DM11 and DM15 showed no clonal variation in all methods.

Preparation of lipid rafts. We used a method that was shown before to be efficient for preparing lipid rafts from C2C12 myotubes (Song et al., 1996), with slight modifications. Briefly, cells grown in 10 cm dishes were washed 2 times with ice-cold PBS containing 1 mM Na-orthovanadate (NaO). After addition of 1.5 ml Buffer A (Na-carbonate 0.5 M, pH 11; inhibitors cocktail as follows: NaO 1 mM, phenylarsine oxide 50 μM, p-nitrophenylphosphate 10 mM, NaF 50 mM, aprotinin 25 μg/ml, leupeptin 25 μg/ml), cells were quickly scraped and suspended using the pipette tip, then homogenized 2 times in a dounce homogenizer and finally sonicated 2 times for 10 seconds. The inhibitors were always prepared freshly and added to buffers just

before use. The total extract (final volume: 2 ml) was quickly mixed with 2 ml 90% sucrose in Buffer B (Mes 25 mM, pH 6.5, NaCl 150 mM + inhibitor cocktail as above) at the bottom of a 13-ml tube and overlaid with 4 ml of 35% sucrose in Buffer C (buffer B + Na-carbonate 250 mM) and then with 4 ml of 5% sucrose in Buffer C, for a total volume of 12 ml. Samples were centrifuged for 17 h at 37'000 rpm in a Sorvall TH-641 rotor at 4°C. 1 ml-fractions were collected from the top and transferred into 3 ml ultraclear-tubes (Beckman). Samples for protein determination (50 µl), cholesterol determination (30 µl) and ganglioside GM1 detection (2 µl) were taken before diluting each fraction with 2 ml Buffer C. Fractions were then centrifuged for 50 min at 100'000 rpm in a Beckman TLA-100.3 rotor, supernatants were discarded and pellets were resuspended in Lämmli-buffer for SDS-Gel electrophoresis and Western blot.

Cell labeling and immunoprecipitation. For AChR stain, cells were incubated 40 minutes with rhodamine-coupled α-BT at 37°C, washed once in PBS at room temperature and then fixed in ice-cold methanol for 7 minutes at -20°C. Conventional fluorescence imaging was done using a Zeiss Axioskop 2 microscope equipped with a Hamamatsu Orcacam digital camera. For quantitation, compact clusters with intensities clearly higher than background and a minimal size of 5 µM were considered as detailed previously (Marangi et al., 2001; Marangi et al., 2002). Clusters were counted in at least 15 fields and experiments were repeated at least 3 times.

For precipitation of MuSK or AChRs, cell lysates were prepared. MuSK-antibodies followed by protein A-sepharose beads or biotin-α-BT followed by streptavidin-beads (Tox-P) were added as described before (Mittaud et al., 2001; Mittaud et al., 2004).

Protein and lipid detection. Antibodies against phosphotyrosine (mixture of PY20 and 4G10); the conserved C-terminus of Src, Fyn, and Yes (src-CT); MuSK; rapsyn (Rap-1); β-dystroglycan; the AChR β subunit (mAb124); and the AChR α subunit (mAb35) were all used in Western blots as described previously (Fuhrer and Hall, 1996; Fuhrer et al., 1999; Marangi et al., 2001; Mittaud et al., 2001; Moransard et al., 2003). Antibodies against α-dystrobrevin-2 were a gift from Dr. D. Blake (Oxford,

United Kingdom). Anti-caveolin-3 (Santa Cruz Biotechnology) was used at 1:2000, anti-flotillin 2/ESA clone 29 (Transduction Laboratories) at 1:1000, and anti-α-tubulin clone DM1A (Sigma) at 1:500. Anti-p75 was as described before (Pun et al., 2002). Densitometric analysis of Western blot signals was performed as done earlier (Marangi et al., 2001) using the software Image J (NIH, USA); the distribution of proteins in the lipid rafts fractions was quantitated by adding up the relative band densities of the raft fractions (4-6) and relating it to the sum of bands in all fractions within each experiment.

For detection of ganglioside GM1, 2 µl of each fraction was applied to a nitrocellulose membrane, blocked with 5% milk in PBS and probed with horseradish peroxidase-coupled cholera-toxin subunit B (10 ng/ml; Sigma; (Hering et al., 2003). To measure cholesterol, 50 µl of each fraction was analyzed with the Amplex Red cholesterol assay kit (Molecular Probes, Eugene, OR (Hering et al., 2003) according to the manufacturer's instruction. Protein concentration was determined using the BCA protein assay kit (Pierce).

Statistical analysis. All values are given as mean ± SEM. Significance was calculated with a t-Test (two-tailed, unequal variance) and is indicated as $p < 0.05$ (*) or $p < 0.01$ (**). In Figure Legends, n refers to the number of experiments.

Chapter 5

Discussion and perspectives

Summary of results and speculations

Synaptogenesis is best understood at the neuromuscular junction (NMJ), where many of the molecular players have been identified (Hall and Sanes, 1993; Sanes et al., 1998). One of the major goals in studying the NMJ is to determine the mechanism by which AChRs are concentrated underneath the motor nerve terminal. Of the molecular players three proteins are critical to the formation of the NMJ endplate-neural agrin, the muscle specific receptor tyrosine kinase (MuSK) and rapsyn. Neural agrin, by activating MuSK and with rapsyn transducing the action causes postsynaptic differentiation events, including clustering, cytoskeletal anchoring and phosphorylation of AChRs (Chapter1).

Protein tyrosine phosphorylation has been increasingly implicated as an important intracellular signaling mechanism for modulating synaptic transmission at the NMJ. It has been suggested that of the non-receptor Src-family kinases (SFKs), Src and Fyn act downstream of MuSK, are activated by agrin and directly cause early AChR β phosphorylation, at least in vitro (Apel et al., 1997; Fuhrer et al., 1997). The functional significance of AChR phosphorylation by Src-class kinases may be in anchoring the receptor to the cytoskeleton. However, clustering of the AChRs can occur in the absence of SFK function suggesting that SFKs are not essential component in the pathways that cause NMJ formation (Smith et al., 2001). Thus in mice lacking Src and Fyn, NMJs are normal around birth, and agrin induces normal AChR clustering and AChR β phosphorylation in cultured *src*$^{-/-}$*;fyn*$^{-/-}$ myotubes (Smith et al., 2001).

While the mechanism described above operate in the initial formation of NMJ, much less is known about the pathways that stabilize the AChR clusters postnatally and mature NMJs to adopt their complex pretzel-shape with the AChRs concentrated at the crests of the postjunctional folds. MuSK is again required (Kong et al., 2004; Hesser et al., 2006) and antibodies against MuSK occur in patients with myasthenia gravis (Hoch et al., 2001), implying that MuSK function keeps NMJ intact. However, some of the pathways for NMJ and AChR cluster maintenance and maturation might

not be required in initial NMJ formation. For example, proteins of the dystrophin/utrophin glycoprotein complex (D/UGC) are important in synaptic stability as seen in mice lacking utropin and dystrophin or the UGC components α-dystrobrevin or dystroglycan (Grady et al., 1997; Grady et al., 2000; Jacobson et al., 2001). In these mice, NMJs form but fail to mature properly. Consistently, cultured myotubes from the knockout mice form normal agrin-induced AChR clusters but on agrin withdrawal the induced AChR clusters rapidly disassemble (Grady et al., 2000). Additional key players are SFKs. In *src*$^{-/-}$;*fyn*$^{-/-}$ myotubes, agrin and laminin induce normal AChR aggregation but clusters disperse rapidly after agrin withdrawal (Smith et al., 2001; Marangi et al., 2002).

To elucidate the mechanism of synaptic stabilization we investigated the role of SFKs in vivo and in vitro. We addressed the mechanism of action of SFKs in postsynaptic cluster maintenance and AChR-protein interactions in *src*$^{-/-}$;*fyn*$^{-/-}$ myotubes and in vivo (Sadasivam et al., 2005). We assessed the role of SFKs in cytoskeletal interactions and their significance in postsynaptic stability (Sadasivam et al., 2005). Further we provide evidence that cholesterol rich microdomains called lipid rafts provide an ideal environment to SFKs to achieve efficient signaling and to maintain protein-interactions and phosphorylations of postsynaptic components (Chapter 4).

We first aimed at investigating the in vivo function of SFKs during later stages of postsynaptic development, since *src*$^{-/-}$;*fyn*$^{-/-}$ mice proved difficult for such analysis due to premature lethality. We show that balanced SFK activity is required for in vivo maintenance of the postsynaptic apparatus (Sadasivam et al., 2005). After electroporation of kinase-inactive Src (Src-AM or Src-K295M) or constitutively active Src (Src-Y527F), the AChR pretzels became fragmented to varying degrees ranging from partial to complete. The relative positioning of the nerve, synaptic nuclei and the AChR clusters are also disturbed and they lie in the same focal plane as confirmed by 3D reconstruction (Sadasivam et al., 2005). In accordance with the postsynaptic changes, the presynaptic nerve terminal sometimes displayed sprouting, as in the case of rapsyn and MuSK deficient mice, suggesting interdependence in signaling and maintenance (Gautam et al., 1995; DeChiara et al., 1996; Kong et al.,

2004; Sadasivam et al., 2005). We assume that such gross changes in the nerve-AChR topology and synaptic nuclei positioning may have arisen due to changes in the postsynaptic cytoskeleton.

Changes in SFK activity lead to downstream pathways affecting cytoskeletal intermediates and organization (Sadasivam et al., 2005). The synaptic rings of α-tubulin synaptic rings were disassembled in Src-AM electroporated fibers in vivo. However alteration in SFK activity did not affect all cytoskeletal proteins. For example, the costameric F-actin organization along myofibers in vivo and the total amount of actin in *src*$^{-/-}$;*fyn*$^{-/-}$ myotubes were unaltered (unpublished observation), suggesting that the effects are specific (Sadasivam et al., 2005). We see that the cytoskeletal link of the AChRs in the *src*$^{-/-}$;*fyn*$^{-/-}$ myotubes is weak under steady state conditions yet strengthened by agrin as determined in detergent extractability assays (Sadasivam et al., 2005). However, the association of rapsyn and dystrobrevin to the cytokeleton appears normal, which might arise from the UGC component utrophin that links to the F-actin network, a process that is probably SFK-independent. Thus AChRs may interact directly or indirectly with other elements of the cytoskeleton through a novel pathway, before and after agrin treatment, and such linkage depends on Src and Fyn (Sadasivam et al., 2005). The agrin-triggered strengthening of the overall AChR-cytoskeletal link may stem from agrin-induced AChR-UGC F-actin interaction.

In order to further investigative the cytoskeletal players in the putative SFK-mediated pathway we looked at additional candidates known to be important in F-actin assembly (Chapter 3). Candidates known to be SFK substrates in other cell types and involved in cytoskeletal dynamics are cortactin, p190RhoGAP and WASp, which ultimately regulate F-actin assembly via the Arp2/3 complex. Reducing SFK activity with inhibitors (PP1, PP2 and SU6656) or studies with *src*$^{-/-}$;*fyn*$^{-/-}$ myotubes show a dramatic decrease in the tyrosine phosphorylation of cortactin at residues PY421 and PY466 and overall tyrosine phosphorylation of p190RhoGAP under steady-state conditions (Chapter 3). These results suggest SFK-dependent F-actin assembly, which may be important in postnatal stability and may partly explain the weak cytoskeletal link of the AChRs in *src*$^{-/-}$;*fyn*$^{-/-}$ myotubes. Another interesting

observation is the synaptic localization of cortactin and p190RhoGAP at endplates, which appeared very similar to the α-tubulin organization. It remains to be determined if electroporation of mutant Src constructs in vivo also causes disassembly of synaptic cortactin and p190RhoGAP. Although the synaptic localization of α-tubulin in vivo and association of cortactin at spontaneous and bead-induced AChR clusters in cultures have been known (Peng et al., 1997), we have shown for the first time a SFK-dependent relevance of these proteins.

In order to further understand the consequences and the mechanism of reduced SFK function we resorted to *src*$^{-/-}$*;fyn*$^{-/-}$ myotubes where co-clustering of postsynaptic proteins with AChRs and AChR-protein interactions can be extensively studied, also at a biochemical level (Sadasivam et al., 2005). Agrin normally recruited proteins of the UGC and rapsyn into AChR-containing clusters in *src*$^{-/-}$*;fyn*$^{-/-}$ myotubes. After agrin withdrawal clusters of these proteins disintegrated strictly in parallel with the AChR clusters. Thus Src and Fyn hold together the postsynaptic apparatus, consistent with the AChR pretzel disassembly in Src-AM-expressing myofibers.

In parallel with the unstable clusters some of the key protein interactions with the AChR were compromised (Sadasivam et al., 2005). The AChR-rapsyn interaction falls apart after agrin withdrawal in *src*$^{-/-}$*;fyn*$^{-/-}$ myotubes. Rapsyn acts as a linker protein connecting the AChR to the UGC complex, and if this interaction is affected, all postsynaptic protein interactions with the AChR disintegrate as seen in our data. Further, SFKs also regulate the overall protein level of rapsyn in the myotubes. In the absence of Src and Fyn, rapsyn protein levels go up two-fold (Sadasivam et al., 2005). AChR and rapsyn are the most abundant of postsynaptic components and the ratio of the two proteins is critical in the metabolic turnover of the AChR and in receptor clustering in myotubes (Gervasio and Phillips, 2005).

We find that SFKs also maintain AChR phosphorylation (Sadasivam et al., 2005). The β-subunit phosphorylation has been implied in the cytoskeletal link of the AChRs during the NMJ formation stages (Borges and Ferns, 2001). We now find that SFKs maintain β phoshorylation and this most likely reflects direct phosphorylation. In *src*$^{-/-}$*;fyn*$^{-/-}$ myotubes, loss of β phosphorylation after agrin withdrawal is paralleled by loss of AChR-rapsyn interaction (Sadasivam et al., 2005). Rapsyn interacts with the

AChRs in the basal state independent of AChR phosphorylation but after agrin addition several experiments have corroborated a tight corelation of rapsyn interaction with the AChR and receptor β phosphorylation (Maimone and Merlie, 1993; Maimone and Enigk, 1999; Bartoli et al., 2001; Huebsch and Maimone, 2003). Firstly in C2C12 myotubes, agrin induces pronounced β phosphorylation and rapsyn binding; secondly, pervanadate treatment causes stronger β phosphorylation and rapsyn binding; and thirdly, the time course of events of agrin-induced β phosphorylation exactly parallels that of AChR-rapsyn binding (M. Moransard and C. Fuhrer, unpublished observations). Thus, increased AChR-rapsyn binding may occur through β-phosphorylation of the AChR either through direct protein interactions or through an intermediate linker. Therefore loss of β phosphorylation of the AChR may diminish AChR-rapsyn interaction, causing postsynaptic disassembly (Sadasivam et al., 2005).

In Chapter 4 we show that cholesterol acts in concert with SFKs within lipid rafts in promoting postsynaptic stabilization and maturation of the NMJ in vivo and in vitro. SFKs are targeted to rafts through their fatty acyl group. Although AChRs and MuSK lack such modification they interact with SFKs (Fuhrer and Hall, 1996; Mohamed et al., 2001); and AChRs and MuSK associate with several postsynaptic proteins that include utrophin (Fuhrer et al., 1999), which binds to F-actin (Winder et al., 1995), and they directly or indirectly associate with several other cytoskeletal proteins. We find that SFKs trigger postsynaptic stabilization by enhancing the association of critical postsynaptic proteins with lipid rafts. In turn rafts allow SFKs to phosphorylate the AChR and maintain AChR-rapsyn interaction.

In Chapter 4 we provide first evidence that cholesterol promotes synaptic stability in vivo. Cholesterol addition to denervated DeSyn gastrocnemius muscle during 12 days prevented postsynaptic disassembly. Furthermore soleus nerve-muscle explants exhibited substantial AChR disassembly which was presented by cholesterol addition. Finally, Botulinum toxin A caused massive nerve sprouting and induction of plaque shaped AChR clusters along sprouts. Addition of cholesterol stabilized and promoted sprout-induced AChR clusters to adopt a pretzel shape. Consistently, cholesterol addition to *src*$^{-/-}$;*fyn*$^{-/-}$ myotubes after agrin withdrawal stabilized AChR clusters.

Conversely, sequestering cholesterol by MβCD treatment (distrupting lipid rafts) mimicked the phenotype observed in the *src*$^{-/-}$;*fyn*$^{-/-}$ myotubes. Firstly, treatment of explants of soleus muscle with MβCD dispersed AChR pretzels, and MβCD disrupted receptor clusters in wild-type myotubes in culture. This perfectly parallels the instability phenotype observed when mutant SFK constructs were electroporated into the soleus muscle of adult mice, and it also agrees with AChR cluster disassembly observed in *src*$^{-/-}$;*fyn*$^{-/-}$ myotubes. We further show that the addition of MβCD to wild-type myotubes reduces rapsyn interaction with the AChR and AChR β phosphorylation, similar to the effects in the *src*$^{-/-}$;*fyn*$^{-/-}$ myotubes. Based on these results we propose a reciprocal relationship between Src and Fyn and rafts in maintaining postsynaptic stability (Chapter 4).

We also found that the proteins incorporated within rafts, especially the AChR and MuSK as well as the amount of cholesterol within rafts, are reduced in *src*$^{-/-}$;*fyn*$^{-/-}$ myotubes. Treatment with cholesterol overcame the instability phenotype in *src*$^{-/-}$;*fyn*$^{-/-}$ cells, by increasing the number of lipid rafts, restoring the lipid-protein ratios in rafts, and by strengthening raft association of AChRs and MuSK (Chapter 4). In summary, rafts are formed due to SFK action and in turn allow these kinases to promote key phosphorylations and protein interactions for maintenance of the postsynaptic apparatus.

Model for SFK mediated signaling at the NMJ

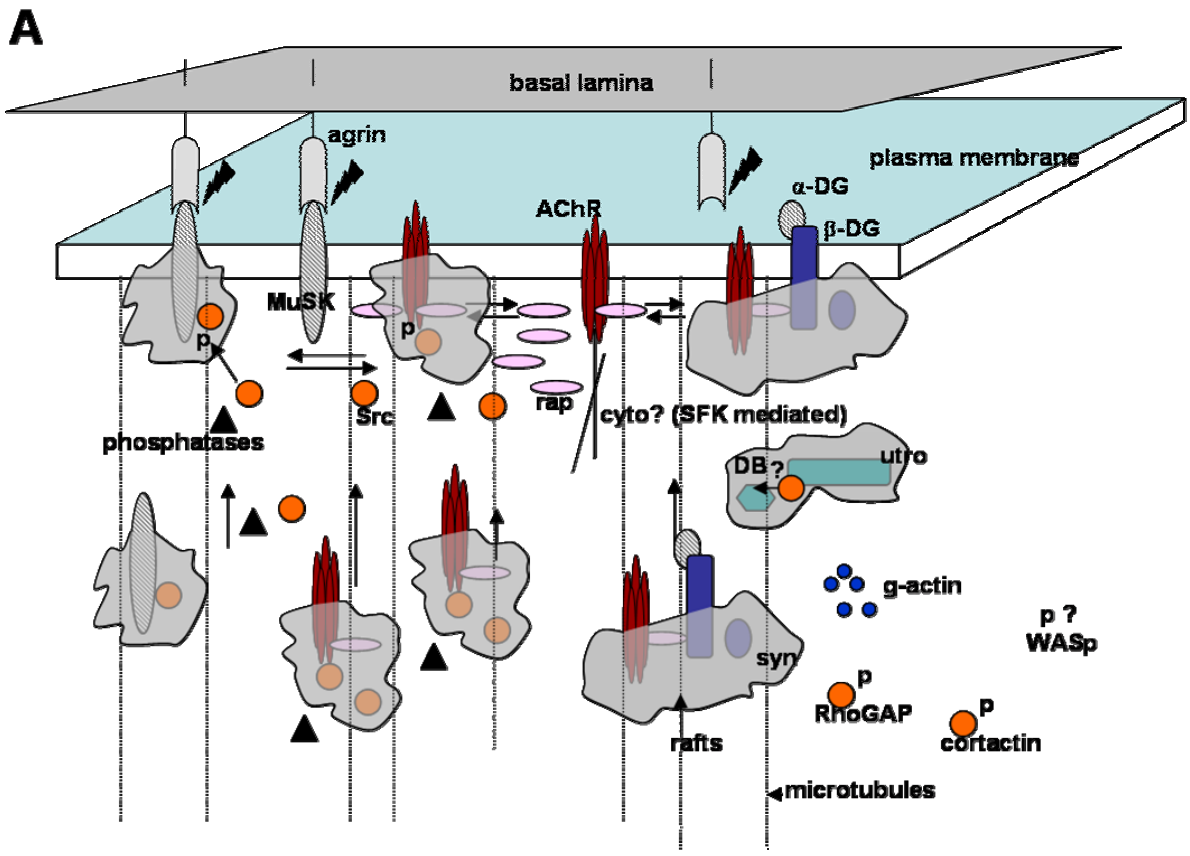
From our studies a interesting model for NMJ stabilization emerges, in which we see a strong dependence on SFKs, cytoskeletal elements and lipid rafts. We propose the following properties of this model. Agrin interacts with MuSK (which could be present in or outside rafts) at the surface, MuSK is activated and in turn triggers a downstream signaling event which leads to the recruitment and clustering of AChRs in a rapsyn-dependent fashion; requiring SFKs for early receptor and MuSK phosphorylation. Intracellularly, SFKs recruit critical postsynaptic proteins like MuSK, AChRs, rapsyn and proteins of the utrophin glycoprotein complex (UGC) into rafts, by binding to them directly or indirectly as suggested by the reduced incorporation of postsynaptic proteins in the *src*$^{-/-}$;*fyn*$^{-/-}$ myotubes. This is followed by

raft migration to the cell surface probably via the microtubular network (Marchand et al., 2002). Rafts are known to be quite heterogenous in their composition and to exist in subfamilies (Simons and Toomre, 2000; Rodgers et al., 2005), like in the immune synapses (Janes et al., 2000; Langlet et al., 2000). Constitutive raft-association of postsynaptic proteins correlates greatly with the existence of pre-assembled complexes at the NMJ during intracellular transport within the secretory pathway (Willmann and Fuhrer, 2002). This may be followed by merging of rafts (microdomains) and accumulation of signaling proteins amplifying the initial signal from the surface receptors and resulting in a cascade of continued raft assembly (macrodomains) and signal amplification. The cascade continues until inhibitory signals are delivered, which attenuate cytoskeletal dynamics, and this could include protein tyrosine phosphatases, which are usually present outside rafts and function to down-regulate signaling of the MuSK receptor complex (Madhavan et al., 2005). All the steps described above are aspects involving NMJ formation, where SFK functions are dispensable as seen in cells lacking *src*$^{-/-}$;*fyn*$^{-/-}$, where AChR clustering and recruitment of the postsynaptic proteins into clusters and protein-protein interactions occur normally in the presence of agrin. However under steady state conditions, SFKs are required to maintain AChR interaction with the cytoskeleton as seen in the detergent extractability assay, although rescued by agrin (Sadasivam et al., 2005). Consistently SFKs were also important in the phosphorylation of cortactin and p190RhoGAP, which are known F-actin regulators (Pantaloni et al., 2000; Higgs and Pollard, 2001) and involve secondary messengers like Cdc42, Rho and Rac that are important in the agrin-induced AChR clustering process (Pantaloni et al., 2000; Weston et al., 2000; Weston et al., 2003).

Most importantly SFKs are required during later stages of development, i.e. postnatal stability and maintenance (Smith et al., 2001; Sadasivam et al., 2005). SFKs appear to stabilize the postsynaptic apparatus within rafts through rapsyn-AChR interaction, AChR phosphorylation and also maintaining cholesterol levels within rafts (Chapter 4). In addition SFKs maintain global rapsyn levels and synaptic α-tubulin rings which might play a role in the intracellular transport of rafts (Marchand et al., 2002; Sadasivam et al., 2005). Interestingly we see a strong correlation between

SFKs and cholesterol within rafts. Sequestering cholesterol from rafts also results in similar destabilizing effects, like the absence of SFKs. Addition of cholesterol rescues the AChR instability phenotype seen in *src*$^{-/-}$;*fyn*$^{-/-}$ myotubes (Chapter 4). We therefore think that rafts are formed due to SFK action and in turn allow these kinases to promote key phosphoylation events and protein interactions for maintenance of the postsynaptic apparatus.

Thus stabilization and maturation of the postsynaptic apparatus are strongly mediated by the members of the SFKs. Defects in SFK activity may stall processes at different levels of synapse development. Examples are defective cytoskeletal assembly, cytoskeletal-mediated transport of postsynaptic proteins in or outside rafts, raft number and recruitment of postsynaptic proteins into rafts. Most importantly at the surface within a cluster, postsynaptic protein interactions are maintained due to SFK action thereby providing sustained signaling at the NMJs.



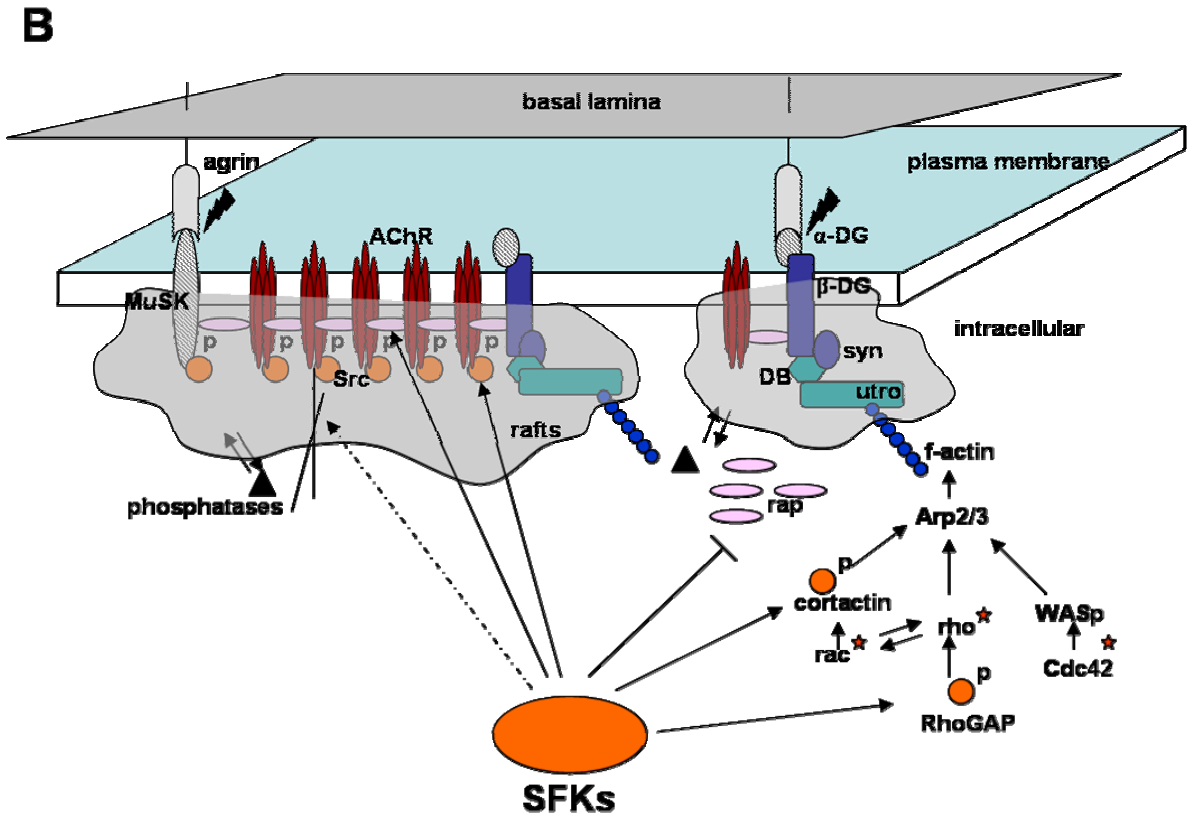


Figure 1. Model of SFK-mediated stability at the NMJ. (A) Activation of MuSK by agrin at the surface triggers an increased delivery of rafts with its associated proteins in the form of pre-assembled complexes via the microtubulin network in an SFK-dependent fashion (although compensated by other mechanisms). For simplicity, the exsistence of rafts in intracellular transparent vesicles is omitted. (B) Eventually rafts coalesce at the surface to form macroclusters within which SFKs maintain protein-protein interactions and rafts integrity. In doing so, SFKs mediate stability by maintaining rapsyn-AChR interaction, AChR phosphorylation and cholesterol levels within rafts. SFKs also negatively control the overall amount of rapsyn protein. SFKs may control the cytoskeletal link of the AChR which could be mediated by α-tubulin binding or by maintaining phosphorylation of cytoskeletal intermediates like cortactin and p190RhoGAP. Cdc42 and Rac activate WASp and cortactin respectively and Rho activity is regulated by p190RhoGAP. Co-operation between Rho and Rac along with Cdc42, is important in the formation of AChR clusters in muscle cells (indicated by the stars). Abbreviation: rap, rapsyn; DB, dystrobrevin; utro, utrophin; α-DG, α-dystroglycan; β-DG, β-dystroglycan; syn, syntrophin; p, tyrosine phosphorylation.

Implications of tyrosine phosphorylation in synapse development

At the onset of postsynaptic differentiation in muscle, neural agrin activates MuSK and triggers a signaling cascade that leads to redistribution of the AChR and directs the assembly of a scaffold for AChR aggregates. Within this signaling cascade lie different effector molecules that positively or negatively regulate in shaping the postsynapse. In the following section I particularly focus on tyrosine kinases as a positive regulator and phosphatases as a negative regulator in shaping the NMJ.

Balancing act: Tyrosine kinases vs phosphatases

The actions of tyrosine kinases and phosphatases have received quite a lot of attention in influencing AChR re-distribution; serving as signal amplifiers, controllers or organizers of postsynaptic differentiation at the NMJ. Even before the discovery of MuSK, tyrosine kinases were thought to be important in regulating AChR clustering because AChR subunits are tyrosine phosphorylated (Qu et al., 1990; Wallace et al., 1991), AChR clusters in situ were labeled by anti-phosphotyrosine antibodies (Baker and Peng, 1993; Peng et al., 1993) and AChR clustering was blocked in the presence of tyrosine kinase inhibitors (Peng et al., 1991; Baker and Peng, 1993; Wallace, 1994). Confirmatory experiments about the significance of tyrosine kinase in AChR clustering came from studies, in which mutant AChRs lacking phosphorylation sites in their β subunit were poorly clustered and attached to the cytoskeleton in response to agrin in muscle cells compared to wild-type AChR counterparts (Borges and Ferns, 2001). Within the agrin-MuSK signaling pathway, agrin causes MuSK tyrosine phosphorylation and activates non-receptor tyrosine kinases, especially Src (Mittaud et al., 2001) and Abl kinases (Finn et al., 2003; Mittaud et al., 2004). Both Src and Abl bind MuSK in an agrin-dependent manner and phosphorylate MuSK; (Finn et al., 2003; Mittaud et al., 2004) blocking Abl activity inhibits agrin-induced AChR clustering (Finn et al., 2003). Briefly stimulating muscle cells with agrin leads to prolonged MuSK phosphorylation, AChR phosphorylation and clustering; and Src and Abl are important for mediating the early and late stages of this effect (Mittaud et al., 2004). Moreover, increased amounts of activitated Src tyrosine kinases associate

with AChRs in biochemical preparations (Mittaud et al., 2004) and co-localize with AChR clusters in cell cultures (Madhavan and Peng, 2005). Cluster formation of AChRs can occur in the absence of SFK function, showing that SFKs are not essential in the pathway that cause NMJ formation (Smith et al., 2001) and AChR clustering is incompletely blocked by inhibition of Abl kinases (Finn et al., 2003), indicating overlapping functions of these and other kinases in AChR aggregation. What appears clear is however, that tyrosine kinases associate with MuSK and amplify and transduce its signal during AChR tyrosine phosphorylation and clustering.

A few years after tyrosine phosphorylation of AChR was implied, the involvement of tyrosine phosphatases in AChR re-distribution was suggested in studies where blocking tyrosine phosphatases by pervanadate increased phosphorylation of MuSK and AChRs and also enhanced the link of the receptor to the cytoskeleton (Wallace, 1994; Meier et al., 1995; Wallace, 1995). This formed the basis for testing whether tyrosine phosphatases mediate dispersal of AChR clusters. In muscle cultures and in Xenopus muscle cells spontaneous AChR clusters called “hot spots” occur, which greatly resemble the AChR clusters generated by the nerve in vivo or those induced by agrin in cultures (Moody-Corbett and Cohen, 1982; Peng, 1986). In the presence of a synaptogenic signals these clusters are dissolved and new clusters are formed. In Xenopus muscle cells inhibition of tyrosine phosphatases blocked dispersal of AChR hot spots by the synaptogenic signal, whereas introduction of a constitutively active tyrosine phosphatase into these cells dispersed hot spots even in absence of a synaptogenic signal (Dai and Peng, 1998). Thus, the agrin-MuSK signaling activates tyrosine kinases that promote AChR clustering and appears to activate downstream tyrosine phosphatases to make AChRs within pre-exisiting clusters available for redistribution.

In addition to cluster dispersal, tyrosine phosphatases can modulate the functional activation of MuSK causing an effect on AChR clustering. When tyrosine phosphatases are partially inhibited such that the AChRs are not completely immobilized, MuSK auto-activation and spontaneous AChR clusters are enhanced (Madhavan and Peng, 2005). Moreover, coupling low level tyrosine phosphatase inhibition with agrin treatment generated enlarged AChR clusters (Madhavan and

Peng, 2005). Accordingly, inhibition of tyrosine phosphatases in bead-treated muscle cells or in nerve co-cultures with muscle causes AChR clusters that are less discrete, with clusters developing beyond the nerve or bead-contact boundaries (Dai and Peng, 1998). These results suggest that the MuSK activation levels are in part set by tyrosine phosphatases. MuSK may activate tyrosine phosphatases to limit its own activity spread. This tight coupling between MuSK, tyrosine kinases and phosphatases could modulate the strength, duration and range of MuSK signalling, which would then help AChRs to cluster maximally at sites where MuSK is set to high activation levels.

An old mathematical-modeling work by Alan Turing revealed that short-range activation and long-range inhibition signals can be combined to generate the spatiotemporal patterns in biological systems (Turing, 1990). One example described was the reaction product that not only catalyses its own production (positive feedback) but also generates an inhibitor signal of its own synthesis (negative feedback loop). In a reaction-diffusion system, this generated highest product levels where the reaction is initiated (Turing, 1990). Applying this to the balancing actions of tyrosine kinases and phosphatases, maximal MuSK activity and AChR clustering occurs at sites where agrin and tyrosine kinase activity are present like in the crests of the junction folds where neural agrin is deposited. However, coupling MuSK activity with that of tyrosine phosphatases would help to ensure that the strength of AChR clustering drops once outside this area and that aneural AChR clusters are dispersed.

AChR clustering machineries and SFK regulation of nicotinic AChRs at peripheral synapses

Recent work has shown that regulation of AChRs by tyrosine kinases such as the Src-family is not limited to the NMJ but also occurs with neuronal nicotinic acetylcholine receptors (nAChRs). These are receptors related to the neuromuscular AChRs but composed of different subunits encoded by a variety of genes (α2-α10; β2-4) (Huh and Fuhrer, 2002). They are formed in peripheral and central nervous system. Like at the NMJ, nAChRs are often clustered in the postsynaptic membrane of cholinergic synapses mediating fast synaptic transmission in the peripheral

nervous system (Huh and Fuhrer, 2002). Examples are the ciliary and superior cervical ganglia, where the nAChRs functionally connect the pre- and postganglionic neurons via cholinergic excitation. In the chick ciliary neurons there are heteromeric nAChRs containing the α3, α5, β2 and β4 subunits and their clustering in the postsynaptic density depends on the signals within the cytoplasmic loop of the α3 subunits and on the postsynaptic functioning of APC protein (Williams et al., 1998; Temburni et al., 2004). This is strictly parallel to the NMJ, where APC is also necessary for AChR clustering (Wang et al., 2003). One APC-interacting protein in the ciliary ganglion is PSD-93, a member of the PSD-95 family of PDZ-domain-containing scaffolding proteins (Temburni et al., 2004). PSD-93 and PSD-95 associate with the nAChRs and interfering with their functions reduces spontaneous EPSC frequency and nicotine-induced long-term phosphorylation of CREB, a transcription factor in ciliary neurons (Conroy et al., 2003). The chick ciliary ganglion also contains homomeric neuronal nAChRs, particularly α7 receptors are present more perisynaptically and yet mediate considerable postsynaptic currents in these neurons (Berg and Conroy, 2002). Spine-like appendages exist on the soma of ciliary neurons, are folded into mats and contain high α7 concentrations in clusters (Shoop et al., 1999). Actin filaments colocalize with the α7 nAChRs (Bruses et al., 2001) and for the retention of somatic spines and stable amounts of α7 nAChRs, the intergrity of the actin cytoskeleton is a requirement (Shoop et al., 2000). Actin dispersal accelerates rundown of α7 nAChR function, whereas actin stabilization diminishes the rundown (Liu and Berg, 1999; Shoop et al., 2000). Besides actin, another mechanism involved in clustering of α7 nAChRs in ciliary neurons is by lipid rafts. α7 nAChRs colocalize with the ganglioside GM1, a marker of lipid rafts and binding partner for cholera toxin; and biochemical raft preparations contain high amount of α7 nAChR (Bruses et al., 2001). Extraction of cholesterol from lipid rafts leads to fragmentation of α7 nAChR clusters into microclusters on the surface of ciliary neurons (Bruses et al., 2001). In summary actin filaments and rafts are part of the machinery that keeps α7 nAChRs in clusters at their perisynaptic location. This represents another striking parallel to the NMJ (see chapter 3 and 4).

Agrin clusters the neuronal nAChRs at cholinergic interneuronal synapses of the superior cervical ganglion in rodents (Gingras et al., 2002), revealing another parallel to the NMJ. Here however, the clustering occurs independent of rapsyn which is expressed at low levels (Feng et al., 1998), although transfection of rapsyn increases that stability of transfected nAChR subunits at the surface of heterologous cells (Kassner et al., 1998). In the superior cervical ganglion the cholinergic synapses form properly in the absence of PSD-93, but nAChRs disassemble once the nerve is denervated (Parker et al., 2004), suggesting that PSD-93 is important for synaptic stability. These data from ciliary and superior cervical ganglia show that both similarities and differences exist in postsynaptic formation, stability and AChR clustering between the cholinergic NMJ, cholinergic autonomic ganglia and CNS glutamatergic synapses.

Neuronal heteromeric nAChRs are also found in neuroendocrine organs like adrenal gland, where receptors of the α3α5β4 type reside on the surface of chromaffin cells and receive input from the innervating cholinergic neurons of the sympathetic nervous system (Di Angelantonio et al., 2003). Binding of either nicotine or acetylcholine to these nAChRs opens the receptor channel, leading to cation influx. The resulting depolarization of the chromaffin cellular membrane opens L-type voltage-gated calcium channels, causing calcium entry and the release of catecholamines via regulated secretory mechanisms into the blood stream (Douglas, 1968; Cheek, 1991).

Studies have shown that SFKs (Src, Fyn and Yes) are highly expressed in the chromaffin cells of the adrenal medulla and that serine/threonine as well as tyrosine kinases influence chromaffin cell secretion (Parsons and Creutz, 1986; Grandori and Hanafusa, 1988; Zhao et al., 1991; Allen et al., 1996; Cox et al., 1996). Tyrosine kinase inhibitors like genistein and tyrophostin 23 block most catecholamine secretion in chromaffin cell when stimulated with nicotine, KCl or calcium ionophores (Cox et al., 1996; Cox and Parsons, 1997). In addition, overexpression of Src reversed the inhibitory effect of vaccinia virus on secretion (Ely et al., 1994). Since SFKs localize to the cell membrane and to the secretory vesicles and are known to regulate secretion

of chromaffin cells, SFKs could act at multiple levels in the pathway leading from nAChR activation to hormone release.

Recent data show that the major regulatory step in this pathway is at the level of nAChRs. Pharmacological inhibitors of SFKs or kinase-dead Src constructs reduce the peak amplitude of nicotine-induced currents in chromaffin cells or heterologous cells that express α3α5β4nAChR (Wang et al., 2004). Conversely, inhibition of tyrosine phosphatases by pervanadate or expression of catalytically activated mutant Src yield enhanced current amplitudes (Wang et al., 2004). Src and Fyn but not Yes co-precipitate with the α3α5β4nAChR and the receptor is tyrosine-phosphorylated on tyrosine residues (Wang et al., 2004), which is very similar to the situation for the muscle AChR (Fuhrer and Hall, 1996). While it remains unclear if the SFKs directly phosphorylate the chromaffin α3α5β4 nAChR, there is this evidence that a balance between SFKs and phosphatases, whose identity is unknown, regulates the activity of nAChRs by opposing actions. SFKs act to activate the receptors where the phosphatases deactivate it (van Hoek et al., 1997). This model is supported by biochemical evidence where SFKs, phosphatases and α3α5β4 nAChRs are present in a large multimeric complex (van Hoek et al., 1997). The role of the phosphorylation of the nAChR itself in this regulatory process, the signals that regulate SFK and/or phosphatases activity, and the significance of nAChR phosphorylation and SFKs in targeting steps or clustering within the chromaffin cells all remain unknown. In summary, while SFKs regulate the postsynaptic distribution of AChRs at the NMJ, they positively regulate the activity of nAChRs in chromaffin cells.

Clustering and SFK regulation of nicotinic AChRs in the central nervous system

The understanding of the trafficking and synaptic clustering of nAChRs in the brain is very limited. Not even the expression and distribution of these receptors in and around CNS synapses are completely known (Huh and Fuhrer, 2002). Of the nAChR subtypes in the brain the most abundant are the heteromeric α4β2 receptors and the homomeric α7 type. The α4β2 receptors are widely distributed throughout the nervous system with highest levels in the thalamus, substantia nigra pars compacta (SNc), ventral tegmental area (VTA) and other brain regions. The α7 nAChR is highly

concentrated in the hippocampus, hypothalamus, olfactory bulb and the amygdala (Seguela et al., 1993). In studies on the rat SNc and VTA, the α4 subunit was detected in a majority of the dopaminergic neurons (Sorenson et al., 1998; Arroyo-Jim nez et al., 1999) and also in some GABAergic cells (Chesselet et al., 1993). The locus could be pinpointed to the postsynaptic density of dendrites of SNc neurons, based on ultrastructural studies. Besides this postsynaptic localization there are also hints to extra- or perisynaptic occurrence of the α4β2 receptor (Sorenson et al., 1998; Arroyo-Jim nez et al., 1999). The α7 nAChRs distribution is pre- or postsynaptic, as well as perisynaptic in the hippocampal neurons (Fabian-Fine et al., 2001; Jones et al., 2004). The α7 nAChRs mediate cholinergic synaptic input to GABAergic interneurons (Frazier et al., 1998; Alkondon et al., 1999) regulating an inhibitory effect within the hippocampal network (Jones and Yakel, 1997). Clusters of α7 nAChRs were visible at postsynaptic localization of dissociated hippocampal neurons (Kawai et al., 2002). The factors influencing the clustering and maintenance of α7 nAChRs in GABAergic interneurons are unclear. Indirect clues such as NMDA receptors and neurotrophic factors like BDNF or NGF act to cluster and maintain α7 nAChRs on hippocampal GABAergic interneuronal cultures (Kawai et al., 2002). Neurotrophins can also influence the mRNA transcription of α7-subunit in PC12 cells (Henderson et al., 1994). Thus, the neurotrphin effect on α7 clustering (Kawai et al., 2002) may primarily originate from a mechanism involving increased receptor expression.

Tyrosine phosphorylation in the brain is known to have an effect on neurotransmitter receptor functioning. Phosphorylation of NMDA receptors in hippocampus has an effect on long-term potentiation (LTP) which can affect learning and memory (Lu et al., 1998). Similarly, α7 nAChRs also have an influence in regulating hippocampal LTP. When acetylcholine is applied to GABAergic interneurons in hippocampal slice cultures and the Schaffer collaerals are activated simultaneously, long-term depression is evoked in the CA1 pyramidal neurons (Ji et al., 2001). Alternatively, when pyramidal neurons are activated with acetylcholine together with Schaffer collateral stimulation, LTP induction is facilitated in pyramidal neurons (Ji et al., 2001). Therefore the question arises if there is any connection between SFK members and the α7 nAChR. Tyrosine kinases of the ErbB family,

when activated by neuregulin, have been shown to cause an increase in the binding of α-bungarotoxin and currents mediated by α7 nAChRs in hippocampal neurons (Liu et al., 2001). But this could stem more from increased transcription of rapid α7-expression rather than the α7 mediated effects on LTP and LTD as described above.

Recent evidence suggests new regulatory mechanisms of nAChR suggesting a balance between tyrosine kinases and phosphatases (Charpantier et al., 2005; Cho et al., 2005). When tyrosine kinases are inhibited by genistein there is an enhanced response of α7 nAChRs on agonist presentation whilst inhibition with tyrosine phosphatases reduced the activity of α7 receptors as suggested in different model systems like Xenopus oocytes, SH-SY5Y neuroblastoma cells and hippocampal interneurons from slice cultures (Charpantier et al., 2005; Cho et al., 2005). In addition, the α7 nAChRs were shown to interact with SFKs and were tyrosine phosphorylated (only the surface receptors); and inhibition of SFKs led to increased α7 receptor activity (Charpantier et al., 2005). Mutant α7 nAChRs lacking cytoplasmic tyrosine phosphorylation sites show enhanced acetylcholine-evoked currents, suggesting that the phosphorylation state of the α7 nAChR determines the responsiveness towards agonists (Charpantier et al., 2005). In summary, a balance between tyrosine kinases and phosphatases whose identity is still unknown seems to regulate the activity of α7 nAChRs by shifting receptors between responsive and unresponsive states. Overall, tyrosine phosphorylation and SFKs mediate a negative effect on α7 activity.

It is still not clear if these changes in α7 nAChR functioning at the surface are purely due to enhanced channel opening or phosphorylation-induced changes that drive trafficking of the receptors to the surface. Although the α7 nAChR was proposed not to undergo rapid exocytosis (Drisdel and Green, 2000) or cytoskeletal changes upon dephosphorylation (Cho et al., 2005), treatment with botulinum neurotoxin decreased genistein-induced α7 receptor potentiation (Cho et al., 2005). Botulinum toxin acts by cleaving SNAP25, a component of the SNARE machinery, and prevents exocytosis. Results show that only 50% of the functional α7 receptor reside in intracellular compartments and the strong increase in α7 nAChR activity induced by genestein (upto 6-fold higher current amplitude) cannot be explained by

trafficking alone (Charpantier et al., 2005; Cho et al., 2005). Moreover, SFK inhibition by genistein and mutation of tyrosine phosphorylation sites did not cause receptor redistribution at the surface of SH-SY5Y cells or dissociated hippocampal neurons (Charpantier et al., 2005) (Wiesner and Fuhrer, unpublished observations); suggesting that the main regulation seems to occur not by receptor trafficking or clustering but by affecting the balance between responsive and non-responsive states of the α7 receptor at the surface.

In summary in contrast to the muscle AChRs at the NMJ, tyrosine phosphorylation of the neuronal nAChRs by members of the SFKs has been not reported to affect receptor distribution, clustering or stability, but receptor function. Thus, although the players are the same, SFKs mediate diverse effects upon muscle and neuronal AChRs in the peripheral and central nervous system.

SFK regulation at other CNS synapses: glutamate receptors

Apart from the nAChR, SFKs have been extensively studied in the regulation of NMDA (N-methyl-D-aspartate) subtypes of glutamate receptors (NMDAR), which mediate fast excitatory transmission at most central synapses. The following section describes how SFKs, in particular Src and Fyn, are crucial for enhancing the NMDAR activity, which in part involves regulation by the opposing actions of protein tyrosine kinases and phosphatases, thereby modulating NMDAR-dependent synaptic plasticity in the brain.

Excitatory transmission in the CNS is primarily mediated by glutamate which is released from the presynaptic nerve terminal from synaptic vesicles via calcium-dependent exocytosis. At most excitatory synapses the presynaptic nerve terminals form synapses on the postsynaptic dendritic spines which contain in their cytoplasmic face the postsynaptic density (PSD). In the PSD there are cytoskeletal proteins that anchor and provide a structural matrix to other key PSD proteins, such as signaling molecules and ionotrophic glutamate receptors, in the postsynaptic membrane. The excitatory action of glutamate is due to the activation of postsynaptic ionotropic glutamate receptors, which are ligand-gated ion channels. There are three pharmacologically and molecularly defined classes of ionotropic receptors which

were originally named according to their preferred agonists: N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methylisoxazole-4-proprionic acid (AMPA), and kainate. For NMDA receptors tyrosine phosphorylation has emerged as a key form of regulation.

Five members of the SFKs are expressed in the mammalian CNS – Src, Fyn, Yes, Lck and Lyn. Within the CNS the first type of channel found to be regulated by the SFKs was the NMDAR subtype of ionotropic glutamate receptors. NMDAR together with AMPA subtypes of glutamate receptors (AMPAR) participate in fast excitatory synaptic transmission in every region of the CNS. Molecular cloning led to the identification of multiple NMDAR subunits which are classified into three subunit families: NR1 (NR1-1, NR1-2, NR1-3 and NR1-4), NR2 (NR2A, NR2B, NR2C and NR2D), and NR3 (NR3A previously called NR-L or χ-1, and NR3B). Native NMDARs are multiprotein complexes comprised of the core NMDAR subunits (NR1, NR2 and possibly NR3), which form the central channel conductance pathway requiring binding of both glutamate and the co-agonist glycine to extracellular sites on the channel complex. The activated channel is permeable to monovalent cations such as Na^+ and K^+ and to divalent cations like Ca^{2+}. The function of NMDARs is diversely regulated by a variety of endogenous regulators binding extracellularly or within the channel pore. In addition, intracellular modulators like serine-threonine and tyrosine phosphorylation, strongly regulate function of NMDARs.

Electrophysiological studies show that NMDAR currents are regulated by a balance of tyrosine phosphorylation and dephosphorylation: inhibiting endogenous protein tyrosine kinase activity (PTK) (Wang and Salter, 1994; Wang et al., 1996) or enhancing phosphotyrosine phosphatases (PTP) activity by exogenous PTP (Wang et al., 1996) leads to suppression of NMDAR currents. Conversely, inhibiting endogenous PTP activity or increasing the PTK activity by introducing exogenous Src increased NMDAR currents (Wang and Salter, 1994). Furthermore in HEK293 cells, recombinant NMDAR activity was enhanced by exogenous Src and Fyn (Kohr and Seeburg, 1996) and also in *Xenopus* oocytes (Chen and Leonard, 1996). These convergent studies suggested that the NMDAR currents are regulated by opposing actions of PTKs and PTPs. Subsequently, single-channel recording of NMDAR

currents combined with increasing PTK activity or inhibiting PTPs was found to increase the NMDAR channel gating with no effect on NMDAR single-channel conductance (Wang et al., 1996).

Kinases of the Src family were implicated as enzymes upregulating NMDAR activity by the use of a phosphopeptide SFK activator (pYEEI peptide), which is a ligand for SFK SH2 domains; and a function-blocking antibody (anti-cst1), which inhibits SFKs but not other PTKs (Roche and Huganir, 1995). The activating peptide was found to increase the activity of synaptic NMDAR currents in cultured neurons (Yu et al., 1997) and in CA1 pyramidal neurons of hippocampal slices (Lu et al., 1998). Furthermore it was shown that specifically Src of the SFK members increases NMDAR-mediated currents. This was done by taking advange of an inhibitory peptide, Src40-58, which is contained within the unique domain of Src and anti-cst1 antibodies, which were raised against the same pepetide (Roche and Huganir, 1995; Yu et al., 1997; Lu et al., 1998). Exogenous Fyn was also shown to increase currents mediated by recombinant NMDARs (Kohr and Seeburg, 1996), but whether endogenous Fyn (or other SFKs present in the CNS), regulates native NMDARs remains to be tested, as the inhibitors that selectively block the activity of each of these SFKs have yet to be developed. However, the Src-specific inhibitors prevent the increase in channel activity produced by the SFK-activating pYEEI peptide (Yu et al., 1997). Src is presumed to cause upregulation of NMDAR channel gating by direct tyrosine phosphorylation of subunits NR2A and NR2B.

NMDARs are not regulated by Src alone but by several other modulators of Src function. Of importance is the striatal enriched tyrosine phosphatase (STEP) family (Oyama et al., 1995), a family of brain-specific, nonreceptor-type PTPs. The $STEP_{61}$ isoform has been found to be a component of the NMDAR complex in spinal cord and hippocampus (Pelkey et al., 2002) and is located downstream of NMDAR function. Application of recombinant STEP to the cytoplasmic aspect of inside-out membrane patches or intracellular application blocked synaptic NMDAR currents, mimicking the effect of inhibiting Src. In contrast, intracellular application of a function-blocking STEP antibody or of a dominant-negative STEP produced an increase in synaptic NMDAR-mediated currents, implying that NMDAR activity is

regulated by endogenous STEP. Both reduction in NMDAR currents by exogenous STEP and increase in NMDAR currents by inhibiting endogenous STEP required Src, since both were prevented by inhibiting Src (Pelkey et al., 2002). Thus STEP fulfills the criteria for an endogenous PTP that regulates the function of NMDARs in opposition to Src. Other regulators of Src activity within the NMDAR complex include positive regulators - tyrosine kinase CAKβ/Pyk2 and protein tyrosine phosphatases PTPα. In addition three other PSD proteins were recently identified to modulate Src within the NMDAR complex: RACK1, H-Ras and ND2 (reviewed by (Kalia et al., 2004).

NMDARs are pivotal for several types of lasting forms of synaptic plasticity in the CNS required for physiological events, including learning and memory. Tetanic stimulation of the Schaffer collateral-CA1 synapses in the hippocampus leads to long-term potentiation (LTP), which is a prominent form of lasting enhancement of synaptic transmission and the predominant cellular model for learning and memory (Kalia et al., 2004). It is hypothesized that during such titanic stimulation, the CAKβ associates with and activates Src (Dikic et al., 1996) via the actions of PTPα which releases Src from its inactive conformation (Zheng et al., 1992; Ponniah et al., 1999). Src activation allows NMDAR function by overcoming the suppression by STEP (Pelkey et al., 2002) and associates with the NMDAR via an adaptor protein, ND2 (Gingrich et al., 2004). This kinase-dependent upregulation is further amplified by the increase in intracellular Na^{+}. Coupled with depolarization-induced reduction of Mg^{2+} inhibition, there is a dramatic boost in the influx of Ca^{2+} through the NMDARs in CA1 neurons which binds to calmodulin (CaM), causing activation of CaMKII. Expression of LTP is ultimately caused due to increased number of synaptic AMPARs in the postsynaptic membrane and/or by phosphorylating existing AMPA receptors causing enhanced channel activity (Kalia et al., 2004). The suppression of Src activity in the signaling cascade may occur by inhibition of Src by H-Ras and RACK1 within the NMDAR complex by negatively modulating SFK activity at synapses. RACK may negatively regulate SFK function by preventing access of SFKs to target sites in the NR2B C-tail; thereby inhibiting SFK mediated upregulation of NMDARs (Yaka et al., 2002). H-Ras in brain slices results in decrease in endogenous

Src activity level and a reduction in tyrosine phosphorylation of endogenous NR2A subunits (Thornton et al., 2003). H-Ras functions as a molecular switch existing in an active GTP-bound form or an inactive-GDP form, but whether the active or inactive form of H-Ras within the NMDAR complex is responsible for the inhibition of Src function has not yet been known.

In conclusion, by upregulating the function of NMDARs, Src gates the production of NMDAR-dependent synaptic potentiation and plasticity such as LTP. Thus Src may be critical for processes underlying synaptic potentiation and plasticity thereby mediating higher order functions like learning and memory within the CNS.

Neuromuscular junction disorders: lessons from natural systems

The safety factor of the NMJ is defined by the ratio between the endplate potential (EPP) generated under normal conditions and that required to achieve the threshold for generation of an action potential. At the human NMJ the safety factor is relatively low, in part due to a low normal quantal content as compared with other vertebrates (Slater et al., 1992). Thus, neuromuscular transmission in humans is particularly susceptible to pathological changes, resulting in myasthenic syndromes. The following section focuses on myasthenic syndromes associated with postsynaptic protein targets like AChRs, MuSK and rapsyn. Genetic or autoimmune disorders associated with these proteins are particularly interesting as SFKs are known to interact with and modulate their functions during synaptogeneis.

The most common myasthenic syndrome is the autoimmune disorder myasthenia gravis (MG), which is caused by antibodies against the nicotinic AChRs of the skeletal muscle (Engel et al., 1999; Nichols et al., 1999; Lindstrom, 2000). In about 20% of the patients with typical symptoms of MG there are no detectable antibodies to AChR, a condition called seronegative MG. However these patients show signs of improvement on plasma transfer or immunosuppressive treatment. When the serum immnuoglobulins (Ig) from these patients were passively transferred into mice it lead to NMJ failure (Mossman et al., 1986). The serum affects the function of AChRs which is transient and associated with AChR phosphorylation (Plested et al., 2002). Several studies indicated the tyrosine kinase MuSK as a likely candidate, to be reconjugated and inhibited by these antibodies; MuSK which forms a part of the receptor for agrin at the NMJ. The immunoglobulin G (IgG) antibodies from patients with serogative MG inhibited agrin-induced AChR clustering, indicating a possible role in agrin-dependent maintenance of the NMJ (Liyanage et al., 2002). It is important to note that there is still a small propotion of MG patients in which no antibody specificity has been defined.

Congenital myasthenic syndromes (CMSs) represent a highly heterogenous group of genetically determined disorders with no known autoimmune basis. Patients are

frequently affected at birth or within the first two years of life, although weakness may only become apparent in adulthood (Engel et al., 1993; Engel et al., 1999). The postsynaptic CMS is divided into two groups: the first consists of kinetic defects of the AChR, such as slow channel (dominant mutations in α, β, δ and ε subunits) and low-affinity fast channel syndromes (recessive mutations in α, β and ε subunits); the second is AChR deficiency syndrome, which is by far the most common of all CMS disorders. Biopsies from these patients show severe reduction in the number of AChRs per endplate and fewer junctional folds. A common feature of this condition is that the endplates consist of multiple, small discrete regions of AChRs and acetylcholinesterase molecules forming an elongated endplate on the postsynaptic membrane (Vincent et al., 1981). Immunostaining shows that the levels of the cytoskeletal protein utrophin are also reduced.

Recent genetic studies have shown that in many cases AChR deficiency is caused by mutations in the AChR ε-subunit gene (Engel et al., 1999). A few have been shown to be in the promoter region (Nichols et al., 1999). However in some of the cases with AChR deficiency, confirmed by analysis of muscle biopsies, genomic screening of patients DNA or cDNA failed to identify any genetic defects of the AChR subunit gene loci. However serogative MG in which antibodies are targeted to MuSK, suggests that mutations in MuSK or in associated proteins involved in the clustering of AChR at the NMJ, could underlie some of the CMS.

Recent studies have indeed identified mutations in rapsyn or MuSK in CMS (Ohno et al., 2002; Chevessier et al., 2004). Three rapsyn mutations were identified: two missense mutations and one frameshift mutation. Expression studies in HEK cells of the missense mutations revealed diminished co-clustering of AChR and rapsyn. Endplate studies showed decreased rapsyn stains and impaired postsynaptic development. AChRs were reduced in number and density and endplate regions were extended over an abnormal length (Ohno et al., 2002). Thus, the rapsyn mutations produced a phenotype that looked very much like the more common AChR deficiency.

Identification of mutations and autoantibodies against key orchestrating proteins like AChRs, MuSK and rapsyn can be of relevance in understanding synaptogenesis from natural model systems. Learning how these specific defects lead to changes in downstream signaling cascades of the agrin-MuSK signaling pathway (for example SFKs) can be challenging in understanding the mechanism of postsynaptic stability and maintenance after development. Most importantly, such understanding at a cellular and molecular level holds promise of future therapeutic approaches for treating these pathologies.

Perspectives

The neuromuscular junction (NMJ) provides an excellent model system to study complexities of synaptogenesis with relative ease and unparalleled technical advantage over other model systems in the brain. Many of the principles in synapse development at the NMJ are at least inpart applicable to the formation of synapses in the brain. The study of synapse formation at the NMJ continues to provide new insights into the understanding of synapse elimination including dynamics at a molecular level that build and maintain the postsynaptic apparatus. In addition, these studies will provide insights into mechanisms underlying muscle diseases, at least for thse pathologies that have their origin at the NMJ.

At the NMJ, synaptogenesis can be broadly classified into several aspects; one involving formation of the postsynaptic apparatus resulting in high density accumulation of AChRs with other postsynaptic proteins; the other is the maintenance of such densities to ensure continued signaling at nerve-muscle contact sites. In our understanding, the molecular players involved in the formation and stabilization of the postsynaptic apparatus are not necessarily the same. For instance, Src-family kinases (SFKs) have emerged as critical players in NMJ stabilization although dispensable for formation.

SFKs act through multiple mechanisms and at different stages of postnatal development to ensure continued signaling at the NMJ. The number and diversity of biochemical pathways that are known to act through SFKs continues to grow. There is abundant evidence that SFKs might be the point of convergence through which a variety of signaling cascades may act in enhancing the function of AChRs. Establishing the molecular pathways and the consequence of such convergence might give new insights into the understanding of postnatal stability mediated via SFKs. With new improved methods in imaging and molecular approaches and owing to the easy accessibility of the NMJ allowing in vivo manipulations in living systems, we

envisage that SFKs might act as a hub for regulating AChRs and will be a pervasive signaling theme for synapse development in health and disease.

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  Sadasivam, G*., Willmann, R., Lin, S., Erb-Vögtli, S., Kong, X.C., Rüegg, M.A., and Fuhrer, C.* 2005. J. Neurosci. 25: 10479-10493

- Cholesterol and lipid rafts stabilize the postsynapse at the neuromuscular junction.

  Willmann, R., Pun, S., Stallmach, L., ***Sadasivam, G.,*** *Santos, A.F., Caroni, C., and Fuhrer, C. 2006. Submitted to EMBO; this paper is currently in revision.*

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